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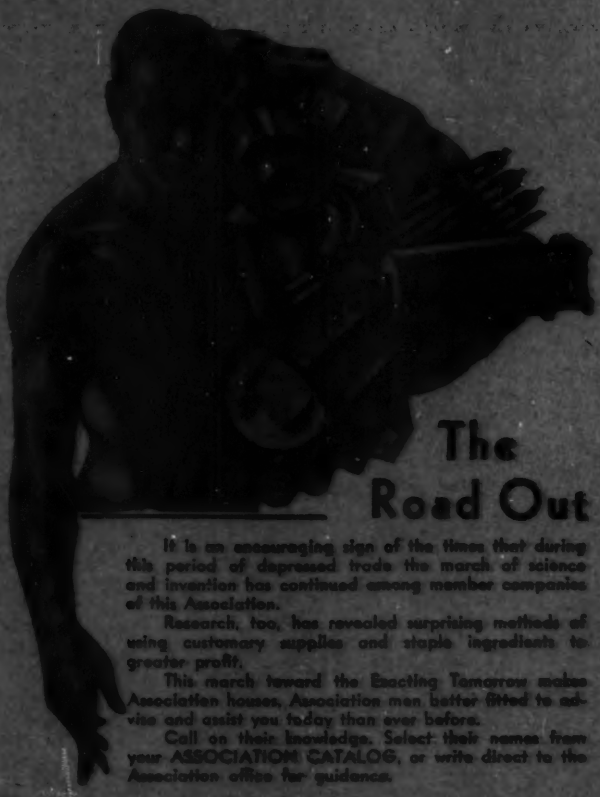

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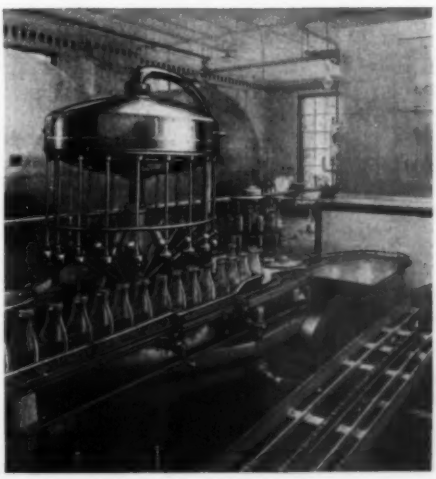

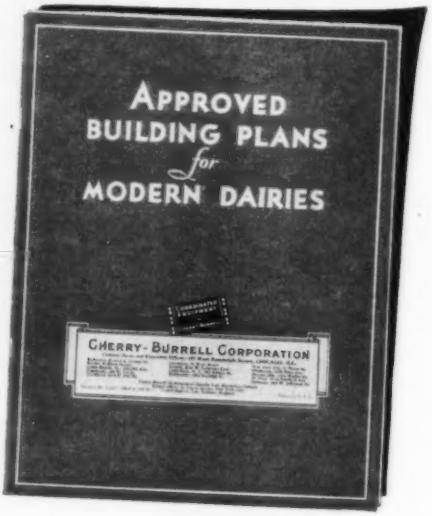

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JOURNAL OF DAIRY SCIENCE

VOLUME XVI MARCH, 1933 NUMBER 2

PROGRESS REPORT OF PASTURE FERTILIZATION AT THE VIRGINIA AGRICULTURAL EXPERIMENT STATION

CHARLES W. HOLDAWAY AND AVERY D. PRATT

Dairy Husbandry Department, Virginia Agricultural Experiment Station, Blacksburg

In April, 1930, the Dairy Husbandry Department of the Virginia Polytechnic Institute, in cooperation with the Department of Agronomy, began an investigation of fertilization of bluegrass pastures. Four 10-acre plots (10A) were laid off in a rolling field on the Smithfield farm about one mile from the campus. This land has been in sod about twenty years and had a fairly good bluegrass sod. There was a fair proportion of white clover mixed in when seasonal conditions favored its growth. A stream, overflow from several springs, flows across all of the plots and provides an abundant supply of spring water at all times. The plots were so laid out that all had the same proportion of higher land and of bottom land along the stream.

FERTILIZATION

One ton of finely pulverized limestone was applied per acre on plots 1 and 2 in March. In addition, 500 pounds of super-phosphate, 160 pounds muriate of potash, and 200 pounds ammonium sulphate per acre were broadcast on the sod. In July another 200 pound application of ammonium sulphate was made, bringing the fertilizer application for the year to the equivalent of 1000 pounds of an 8–8–8 mixture. Plots 3 and 4 served as check plots and were not fertilized. No seed was applied.

MANAGEMENT

Each 10-acre plot was fenced with woven wire to prevent the cows from reaching through into the adjoining lot. One of the fertilized plots (No. 2) was subdivided by fencing to make three lots of equal size, 3⅓A. each. One of the unfertilized plots (No. 3) was similarly subdivided. The cows were rotated from one subdivision to another every third day, thus pasturing for three days and then giving six days for the pasture to reestablish itself. With this arrangement we are able to test the merits of rotation on both fertilized and unfertilized land.

Received for publication May 18, 1932.

Milking cows were pastured on each plot as soon as there was sufficient grass; additional animals were added or some were removed whenever necessary, the intention being to have all plots pastured so as to keep the grass fairly short at all times. Some dry animals were used when sufficient milkers were not available.

Gates lead from each plot into a lane through which the cows pass to the milking shed. All cows were milked twice daily.

No grain was fed to any of these cows while on pasture.

All cows were weighed three days in succession when going on and again when coming off from a plot, these weights being averaged to determine gain or loss in weight, and also to determine the maintenance requirements.

Each milking was weighed individually, while Babcock tests and lactometer readings were made every ten days.

Salt blocks were placed in the plots where they were available at all times.

1930 PASTURE SEASON

The 1930 pasture season will be remembered as the drought year.

Cattle were first placed on the plots May 7. The rainfall was rather scant through June. The cows were removed from all lots July 1, when the second application of ammonium sulphate was made. No more rain fell and the cows were not returned to the plots during the remainder of the season. In many places large patches of sod were killed and could be scraped up by the foot, the grass roots cleaving loose from the soil. By mid-season of 1931 bluegrass had reclaimed these areas so that they could hardly be located.

Since rainfall was not plentiful before the 1931 pasture season, we may assume that much of the fertilizer applied was retained in the soil and doubtless affected the 1931 pasture yields.

The white clover was practically all killed off by this extreme drouth. Since the 1930 results apply for less than two months and because the fertilizer had little opportunity to affect the pastures, no results will be reported here for that season.

1931 PASTURE SEASON

The effect of fertilization was evident very early in the earlier greening of the fertilized plots 1 and 2, and in a heavier stand of plants. On April 14 five cows were placed on each of plots 1 and 2. On April 21, two more cows were added to each plot. By this time the unfertilized plots were sufficiently advanced that five cows were placed on each. By May 13 there were twenty cows on each of the fertilized plots, but only the original five on each of the unfertilized.

By July 1, when all animals were removed for the second nitrogen application, the two fertilized plots had furnished 1339 actual pasture days, while the unfertilized had provided but 436 pasture days, or one-third as

much. A short dry season prevented returning the cows until July 13. Additions and withdrawals were made as necessity demanded until November 9, when the last animals were removed.

After October 1, the two fertilized pastures provided a total of 489 actual pasture days, while the two unfertilized provided but 216 days.

This shows very definitely that fertilization makes it possible to avoid late spring barn feeding and early fall barn feeding.

PRESENTATION OF DATA

To obtain the total pounds of total digestible nutrients as shown in table 1, produced by the four plots, the following methods were used. The average weight of each cow was multiplied by the number of days she was on a plot and divided by 1000 to obtain the number of pasture days for a 1000 pound cow. This is shown in table 1 as pasture days. On the basis of the Morrison requirement, the pounds of total digestible nutrients for maintenance were calculated.

TABLE I

Summarization of pasture data for the pasture year (April 14–Nov. 9) 1931

	PASTURE PLOTS			
	1 Fertilized—cattle not rotated	2 Fertilized—cattle rotated	3 Not fertilized—cattle rotated	4 Not fertilized—cattle not rotated
Pasture days (1000 pound cow)	1984.3	2159.5	1199.5	1134.4
Pounds T.D.N. required for maintenance	15725.86	17114.31	9506.47	8990.07
Pounds gained	732	1579	962	1099
Pounds T.D.N. required to produce this gain	2379.00	5131.75	3126.50	3571.75
Milk produced (converted to 4% fat)	35141.0	26280.7	13080.5	14534.7
Pounds T.D.N. required to produce this milk	11526.25	8620.07	4290.40	4767.38
Total pounds T.D.N. produced	29991.11	30866.13	16923.37	17329.20

The animals were weighed three successive days and the average used to determine gains or losses. Taking Armsby[1] as our authority that the energy requirement in addition to maintenance for the production of a pound of gain on a mature animal is 3.25 Therms and knowing that one pound di-

[1] The Nutrition of Farm Animals, Armsby, 1922, Macmillan Co., New York. Appendix, Table III.

gestible nutrients is roughly equivalent to one Therm, the equivalents are obtained for the gains of the four groups.

The actual milk and fat production for each group was converted by the formula of Gaines and Davidson[2] to an amount of 4 per cent milk that is equivalent on an energy basis to the actual production. This formula (0.4 times the pounds of milk plus 15 times the pounds of fat equals an equivalent amount of 4 per cent milk) makes group comparisons possible and simplifies calculation of the total digestible nutrients required for milk production.

Multiplying the average requirement (0.328 pounds total digestible nutrients) by the pounds of 4 per cent milk produced, yielded the pounds of total digestible nutrients required for production as shown in the table.

INTERPRETATION OF DATA

First, consider the results of rotating the animals. Plot 2 provided 875 pounds more total digestible nutrients than Plot 1. However, Plot 4 yielded 406 pounds more total digestible nutrients than the unfertilized plot (No. 3) on which the cattle were rotated. If the production of both plots on which the cows were rotated be matched against those on which rotation was not practiced, an advantage of 469 pounds total nutrients, or only 1 per cent of the whole, in favor of rotation is found.

If in the same manner we compare both fertilized with the unfertilized plots, we find a greater production of 26,604 pounds total digestible nutrients, which is 78 per cent increase due to fertilization.

Five dollars rental per acre is paid on this land. The yearly cost of fertilization was $17.50 per acre at current local prices, or $350.00 for the plots 1 and 2. The feed cost of a pound of butterfat on the unfertilized plots was 9.05 cents and on the fertilized 18.31 cents, making the cost of the fat resulting from fertilization 25.88 cents per pound. This was approximately the retail value.

If the gains in weight for the fertilized and unfertilized be converted to an equivalent amount of milk on the basis of the amount of digestible nutrients required for the production of each, we find:

Fertilized	22,898.6	pounds 4 per cent milk
Unfertilized	20,421.5	pounds 4 per cent milk

Adding this to the actual production and recalculating, we find a feed cost of 7.02 cents for fat produced on the unfertilized and 13.34 cents for the fertilized. This made the fat equivalent due to fertilization cost 24.12 cents. On this basis the feed cost per hundredweight of 4 per cent milk was

[2] Gaines, W. L., and Davidson, F. A., Relation between percentage fat content and yield of milk. Ill. Exp. Sta. Bul. 245, 1923.

28.1 cents for the unfertilized, 53.4 cents for the fertilized, and 96.4 cents for the milk resulting from fertilization. This evidently is still within the limit of profitable production in fluid milk areas.

On the basis of authoritative milk analyses, the following losses in fertility by removing this amount of milk are calculated:

	NITROGEN	FERTILIZER ELEMENTS (POUNDS)	
		Phosphorus pentoxide	Potassium oxide
Fertilized plots	300	104.4	104.4
Unfertilized plots	135	46.9	46.9

The application of the elements nitrogen, phosphorus, and potash to the fertilized area was 1600 pounds. This of course leaves a large reserve in the soil, the results of which will probably be evident for at least a five-year period if fertilization were now discontinued.

In conclusion it seems that for the first season the cost of milk or fat due to fertilization is dangerously near the market value of the product, when no account is taken of the residual effect on the soil. When it is realized that but a small proportion of the added fertility was removed, it would seem that the increased pasturage that should follow for several years is sufficient justification for this financial outlay. No attempt has been made to review the extensive literature on this subject.

PROTEINS OF THE WHEY FRACTION IN MILK FROM NORMAL AND ABNORMAL UDDERS

F. S. JONES AND RALPH B. LITTLE

Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.

It is known that during normal lactation milk contains relatively constant quantities of protein. The bulk of the protein constituents is made up of casein but when this is precipitated by rennet or weak acids there remain in the whey small quantities of other proteins. These make up, according to Van Slyke (1), about 0.8 to 1.0 per cent of the total solids of milk. The proteins of whey have been obtained in a high degree of purity by Osborne and Wakemen (2) who designate 3 types, lactalbumin, lactoglobulin, and an alcohol soluble protein. Both by chemical and immunological methods Crowther and Rastrick (3), and Wells and Osborne (4) have shown that the lactoglobulin cannot be differentiated from the blood globulin although blood albumin differs from lactalbumin. Analyses of whey proteins, as usually given, are—albumin, six mgs. per cc.; globulin, two mgs. per cc. (Van Slyke, 1). Jones (5) was able to show that milk from normal cows, probably because of its globulin content, reacted at definite levels with a cow serum precipitin and that when the udder became inflamed smaller quantities of milk reacted with the blood serum precipitin, indicating increased blood serum antigens in the milk.

The methods commonly employed for estimating the protein fraction are tedious and a simple procedure would be advantageous. This paper deals for the most part with a method of estimating the proteins of whey. In addition we have attempted to show by means of precipitation tests the relationship between certain whey proteins and those of the blood serum.

METHODS

In order to determine volumetrically the quantity of whey proteins the following procedure was used. One part of milk and two parts of 0.9 per cent NaCl are mixed and sufficient normal HCl is added, with constant stirring, to coagulate the casein. A clear or slightly opalescent whey is obtained after filtration through fine filter paper. One cc. of the filtrate, 1 cc. of distilled H_2O, and 2 cc. of 20 per cent trichloroacetic acid are placed in capillary centrifuge tubes. The tubes used by us have a capillary 2.5 mm. in diameter and 20 mm. in length. Centrifugation at 2500 R.P.M.

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* The authors are indebted to Dr. M. L. Anson, of The Rockefeller Institute, for cooperation during this work.

for ½ hour is sufficient to pack the precipitate. The length of the column may be measured under the low power of the microscope by means of a divided ocular and mechanical stage micrometer. The result when multiplied by three can be expressed in mm. of precipitate per cc. of milk. When abnormal milk is employed it is advisable to use only fractions of 1 cc. of filtrate since too much protein is apt to clog the constriction of the tube.

In order to determine the character of the protein, precipitin was prepared by the injection of rabbits with normal cow serum. When 0.1 cc. of antiserum would definitely react with 1/40960 cc. of cow serum, the animals were bled and the serum stored (0.1 cc. of serum was always employed in making the tests with diluted milk).

For quantitative determinations of the proteins, the colorimetric method of Greenberg (7) was used. In a few instances total nitrogen determinations were made by the Kjeldahl method. The milk was treated as already described and the total quantity of protein determined. Since it was essential to find the ratio of proteins precipitated by ammonium sulphate to the total protein, the following was employed: 1 volume of whole milk was rapidly stirred while 1 volume of saturated ammonium sulphate was slowly added. The mixture was filtered and an equal volume of 20 per cent trichloroacetic acid was added to the clear filtrate. The precipitate, packed by centrifugation, was dissolved in sufficient N/5 NaOH to equal the original volume of milk, and the quantity of protein determined. This quantity was subtracted from the total and the difference regarded as the fraction precipitated by ammonium sulphate.

As the problem developed it became essential to indicate whether or not the total quantity of precipitable material could be considered as protein or split products. Hiller and Van Slyke (8) by means of precipitants in various concentrations were able to separate proteins from their split products. They showed that tungstic acid or 20 per cent trichloroacetic acid removed both proteins and their derivatives. In some experiments, not reported in detail in this paper, it was found that if an equal volume of 20 per cent trichloroacetic acid was added to whey and the solution filtered there remained in the filtrate only a trace of materials which could react colorimetrically. When the concentration of trichloroacetic acid was lowered to 5 per cent the filtrate always contained substances which reacted colorimetrically. By means of precipitation with 5 per cent trichloroacetic acid it was possible then to determine the quantity of both whole proteins and split products.

EXPERIMENTAL

In order to show that the volumetric determination is sufficiently accurate for our purposes the following experiment is cited.

Experiment 1.—Casein from 10 cc. of normal milk, to which 20 cc. of 0.9 per cent NaCl was added, was precipitated with normal HCl and removed by filtration. One cc. of filtrate, after the addition of 20 per cent trichloroacetic acid and centrifugation, gave a column of protein 1.9 mm. in length. From this it was calculated that the total precipitable protein in 10 cc. of milk equaled 57 mm. Two cc. of cow serum was mixed with 20 cc. NaCl and handled as above. One cc. of the filtrate when precipiated with trichloroacetic acid gave 11.2 mm. of precipitate. Thus, the total precipitate from 2 cc. of blood serum was 246 mm.

Ten cc. of the milk, to which 2 cc. of cow serum had been added, was mixed with 20 cc. of NaCl and the solution filtered. One cc. of the filtrate after precipitation of the casein gave 11 mm. of precipitate after the addition of trichloroacetic acid and centrifugation, and from this it was calculated that the milk and serum mixture contained 320 mm.

The total precipitate obtained from milk, 57 mm., plus that of the serum equivalent to 246 mm. equals 303 mms. against 320 mms. actually found in the mixture of milk and serum. This argues that for our purposes the method was sufficiently accurate.

In other experiments where as little as 0.5 or 1 per cent of cow serum was added to milk, increases in the quantity of precipitable protein were readily detected in the whey.

If then the method was of sufficient accuracy to detect the addition of as little as 0.5 to 1 per cent of serum to milk, it should show abnormalities in the whey protein volume in milk drawn from abnormal udders. In order to establish this premise a number of examinations were made, both of normal milk and that obtained directly from cases of mastitis. For the sake of completeness not only the volumetric determinations are given but these measurements have been correlated with the quantity of proteins as determined by the colorimetric method. In addition, the proportion of protein precipitated by half saturated ammonium sulphate to the fraction not so precipitated is given. For convenience the former is referred to as the globulin fraction and the latter as albumin. The results are given in Table I.

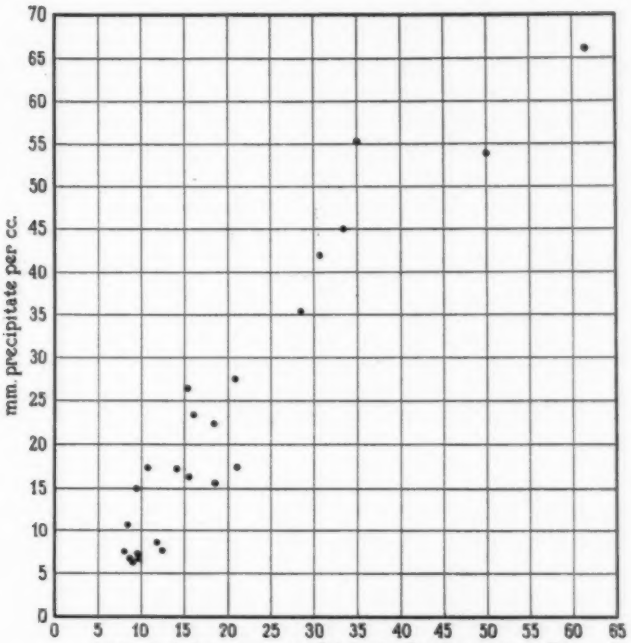
When a portion of the data given in table I is analyzed, as when the volumetric determination of precipitate given in mm. per cc. of whey is plotted against mgs. per cc. of protein, a fair degree of correlation is afforded as shown in graph I. The volume of precipitable material then can be said to correspond in general with the total quantity determined colorimetrically.

In general the albumin-globulin ratio is dissimilar to the previously reported findings. In the 8 normal milks, according to other workers, an albumin-globulin ratio of 3:1 was expected; however, from the data in general the ratio is 4.5 to 5.0. In mastitis, aside from the definite increase

TABLE I

The volumetric determination of whey proteins compared to quantitative colorimetric determinations

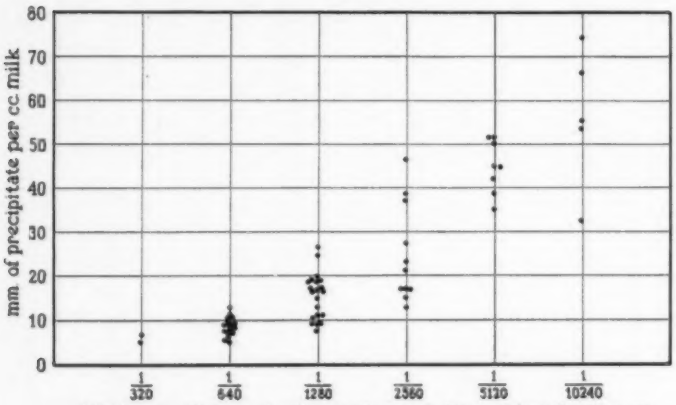
KIND OF MILK	VOLUME OF PRECIPITATE PER CC.	MILLIGRAMS OF PROTEIN PER CC.	ALBUMIN GLOBULIN RATIO	KIND OF MILK	VOLUME OF PRECIPITATE PER CC.	MILLIGRAMS OF PROTEIN PER CC.	ALBUMIN GLOBULIN RATIO
			4.0				7.4
Jersey normal	6.3	9.1	5.1	3349 mastitis	55.8	35.3	27.9
			4.8				5.4
Guernsey normal	8.7	11.8	7.0	1030 mastitis	35.4	28.4	23.0
			5.8				5.9
Holstein normal	7.8	12.4	6.6	2849 mastitis	22.4	18.4	12.5
			3.9				4.6
Holstein normal	6.6	8.6	4.7	8694 R.F. normal	10.8	8.3	3.7
			4.3				10.2
Guernsey normal	7.2	9.8	5.5	8694 L.F. mastitis	42.0	30.8	20.6
			4.3				8.5
Mixed milk normal	6.9	9.8	5.5	8048 L.H. mastitis	15.6	18.5	10.0
			13.1				8.1
U. 96 mastitis	66.0	61.5	48.4	F. 46 R.H. mastitis	16.2	15.3	7.2
			13.1				5.2
A. 52 mastitis	53.8	50.0	36.9	F. 46 L.H. mastitis	17.1	14.0	8.8
			4.4				7.4
2237 mastitis	26.4	15.1	10.7	8048 L.F. mastitis	27.6	21.8	14.4
			4.9				6.1
2739 mastitis	45.6	33.3	28.4	74 R.F. mastitis	21.0	17.4	11.3
			4.6				4.7
111 mastitis	23.4	16.0	11.4	74 L.H. normal	7.5	8.0	3.3
							3.7
				60 R.F. mastitis	15.0	9.4	5.7
							3.7
				60 L.H. mastitis	17.4	10.7	7.0



GRAPH I

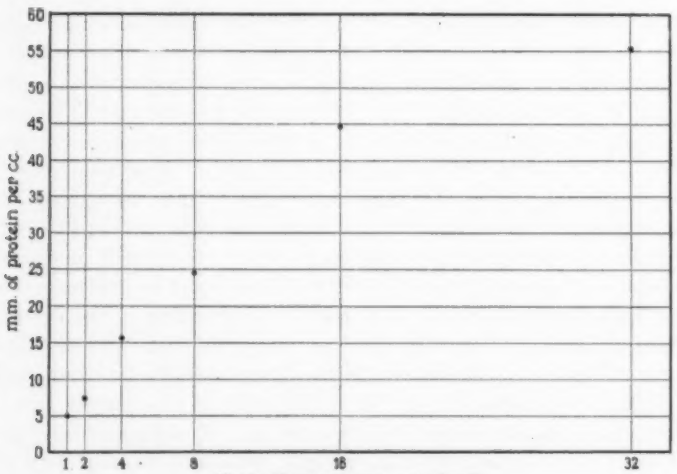
in whey protein, sharp differences in albumin-globulin ratio are indicated in table I. In general, the bulk of the protein behaves as globulin. This is particularly true when the total protein is considerable since in such cases the albumin-globulin ratio is 1:2, 1:3 and in one instance 1:5+. In no case is there a proportionate increase of albumin as compared to globulin.

The question of the origin of the whey protein is important. According to the present knowledge the lactalbumin differs from the albumin of the blood, whereas the milk globulin is essentially the same as that of the blood. Furthermore, diluted normal milk reacts when mixed with a cow serum precipitin. If then the proteins which we have found in greater quantities in mastitis milk are foreign to the udder and originate in the blood, then the ability of milk to react with blood serum precipitin should be enhanced. This actually proved to be the case in the 65 samples of milk drawn directly from normal and abnormal udders. In graph II the results have been recorded, the ordinate comprising the total mm. of precipitate per cc. and the abscissa the minimum dilution of milk definitely reacting with 0.1 cc. of anti-cow serum.



GRAPH II

The data indicate a definite correlation between the volume of precipitable protein in the whey and the property of the milk to react with blood serum precipitin. When the difficulties of obtaining an exact end point for precipitin tests and the considerable space between each dilution are taken into consideration the results are indicative. If the average of the various measurements plotted against reciprocals of the dilution and the 1:320 dilution is regarded as containing one antigenic unit and, the antigenic unit is doubled on each subsequent dilution, the correlation as



GRAPH III

plotted in graph III is sharper and the points fall more nearly on a straight line.

It might be argued that the methods employed for the volumetric estimation of protein and the colorimetric method indicated not only whole proteins but protein split products as well. This seemed altogether likely since in the first method the precipitant is an equal volume of 20 per cent trichloroacetic acid and in the colorimetric procedure the comparisons are made against a tryosine standard. This phase of the question is considered in table II where both the total quantity in whey and that remaining in the filtrate after precipitation with 5 per cent trichloroacetic acid is determined colorimetrically.

TABLE II

Quantitative determination of protein and derivatives precipitated by 10 per cent and 5 per cent trichloroacetic acid

	TOTAL IN WHEY MGS.	NOT PRECIPITATED BY 5% TRICHLOROACETIC ACID		TOTAL IN WHEY MGS.	NOT PRECIPITATED BY 5% TRICHLOROACETIC ACID
Normal 1	9.1	3.9 mgs.	Mastitis 4	23.1	8.5 mgs.
" 2	13.0	5.7 "	" 5	50.5	6.5 "
" 3	12.6	5.7 "	" 6	24.2	8.8 "
" 4	13.0	5.0 "	" 7	26.4	7.7 "
" 5	10.7	5.0 "	" 8	43.6	12.0 "
" 6	9.2	3.4 "	" 9	40.0	9.7 "
" 7	13.0	3.6 "	" 10	42.0	10.9 "
Mastitis 1	19.0	4.8 "	" 11	25.3	8.0 "
" 2	20.3	3.9 "	" 12	39.3	7.4 "
" 3	40.7	5.4 "	" 13	22.9	6.1 "

In general the normal milk whey averages about 11.5 mgs. per cc. Approximately 60 per cent of this might be regarded as whole protein since it is precipitated by 5 per cent trichloroacetic acid. This indicates that under normal conditions the whey fraction contains a considerable quantity of split products.

It might be expected that during udder inflammation, when the protein is increased, the quantity of material left in solution after treatment with 5 per cent trichloroacetic acid would be considerable but the results obtained do not bear out this view. Although there is an increase in this material, it averages less than one-quarter of the total.

DISCUSSION

We have called attention to a method whereby the proteins of whey may be readily measured. Unfortunately the volumetric procedure is not satis-

factory as a quantitative measure of various fractions of the whey proteins. Unreported experiments indicate that there is considerable difference in the degree of packing between the globulin fraction and the albuminous portion. The latter forms a compact deposit but the globulin portion packs less tightly. This fact, however, is of distinct advantage since the admixture of a little additional globulin is readily detected.

Over a period of several months milks of many normal cows have been examined by the volumetric procedure. The normal reading in centrifuge tubes having a capillary diameter of 2.5 mm. has fallen between 6 and 9 mm. per cc. corresponding in general to 8 to 12 mgs. of protein per cc. as determined quantitatively. On the average a little less than one-half of the total protein falls into the fraction not precipitated by ammonium sulphate and the remainder into the globulin portion.

In many cases of mastitis there is a sharp rise in the whey protein level as indicated by the volumetric method and substantiated by colorimetric determination. Here the great bulk of the increase lies in the globulin portion since the ratio of globulin to albumin is increased one, two or more times. The fact that milks whose whey proteins are excessive react more readily with a blood serum precipitin suggests that the protein originates in the blood serum, and as the result of inflammation the capillaries and udder structures become permeable to blood protein.

The difference in the proportion of albumin to globulin in milks from inflamed udders is of interest. Howe and Sanderson (9) in their studies on the blood of cows found that the ratio of albumin to globulin was about 5 : 3. It might be presumed that under abnormal conditions when blood serum entered the udder, a proportionate quantity of each fraction would be found. The data in table I show that this is not the case. So far as our present knowledge goes it seems hardly plausible that vascular permeability would be greatly increased for one type of protein (globulin) and not for albumin. In all probability both pass into the milk under abnormal conditions and it seems possible that the albuminous portions may be so changed as to lose their original identity. The recent experiments of Fischer (10) bear out this view. He noted that the addition of antithrombin to blood albumin so changed its behavior that in many respects it resembled globulin. It is not unlikely that similar changes may take place in the inflamed udder.

The observations where various concentrations of trichloroacetic acid were used as precipitants are of interest. If, for comparison, the substances precipitated by 5 per cent trichloroacetic acid are regarded as whole proteins, nearly half of the total precipitable material in normal whey might be regarded as protein split products. It might be assumed that during inflammation much more of this material would be encountered. It is true

that there is an increase in such substances but in no instance have they been found to be more than 30 per cent of the total. On the average the ratio of such substances to the total has been less than that of normal milk. The facts substantiate the view that the increase during inflammation is due to admixture of blood protein and not to broken down udder tissues.

SUMMARY

A volumetric method of determining the quantity of proteins other than casein in milk has been described. It was possible to correlate volume of protein precipitate with actual quantity of protein as determined colorimetrically. The results indicate that normal milk contains a fairly uniform quantity of such proteins. Under abnormal udder conditions they are usually increased. The observations suggest that the proteins found in the milk during mastitis originated in the blood. In addition, differences in the albumin-globulin ratio between normal milk and that from abnormal udders have been found.

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ACID MILK IN BOVINE MASTITIS

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The fact that milk drawn from normal udders is slightly acid (pH 6.6) is well known. In many cases when the udder becomes abnormal, as the result of bacterial invasion, the secretion is more alkaline and the pH approaches more nearly that of the blood, as first reported by Baker and Van Slyke (1). This fact has been fully substantiated by many workers, and the use of such indicators as brom thymol blue, bromcresol purple and phenol red as rapid means of detecting mastitis has become a common practice.

In a previous paper (2) we recorded, among a number of cases, three that differed from the others in that the milk was distinctly acid, whereas the bulk of the samples were of normal reaction (pH 6.6) or slightly more alkaline (pH 6.8 to 7.4). Since the phenomenon was wholly unexpected in milk drawn directly from the udder it seemed worthy of study.

Occasional specimens of acid milk from bovine mastitis have been encountered by Gloy and Bischoff (3). Baker and Breed (4) also noted the phenomenon and related it to carbohydrate fermentation by the accompanying organisms. It is not clear whether or not the hyperacidity was found in milk drawn directly from the udder and examined at once or resulted from bacterial multiplication after the milk was drawn.

Since in our first observations the milk was drawn directly from the udder and examined within an hour, the evidence indicated that we were dealing with a type of reaction that occurred within the udder. The acidity then was not the result of bacterial growth and carbohydrate utilization in the milk outside of the mammary gland but the acidity came about in the udder itself. With the idea of studying the phenomenon in greater detail a number of cases were examined. In this connection we wish to acknowledge our indebtedness to Dr. J. G. Hardenburg of the Walker-Gordon Laboratory for his cooperation in this work.

THE CLINICAL ASPECTS OF CASES HAVING ACID MILK

In all, 9 cases of mastitis associated with distinctly acid milk have been encountered among a series of over 440. The records fail to indicate uniformity in previous attacks; some occurred after repeated attacks in various quarters, others had no such record. In the main the disease resembled the usual acute mastitis, the quarter being swollen and the milk sero-purulent.

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The milk had a characteristic appearance, the color was yellowish-white and on standing readily separated. The sediment was composed of flocculent yellow material which usually made up one-quarter or more of the total. The supernatant was only slightly turbid and serous and contained little fat.

In general it can be said that infections accompanied by the secretion of acid milk are apt to injure the udder to such an extent that the animals are no longer profitable as milk producers.

MATERIALS AND METHODS

Throughout this work the milk was obtained in sterile bottles directly from the udder. Its pH was determined colorimetrically[1] within a short time and when the reaction was acid additional samples were collected immediately. In every instance samples were drawn at the succeeding milking and at intervals thereafter.

For bacteriological examinations the milk was diluted in broth in series beginning at 10^{-1} and ending at 10^{-7} and 1 cc of the various dilutions plated with 12 cc. of veal infusion agar. The colonies were counted after 48 hours of incubation at 38°C.

We attempted to estimate the number of leucocytes by the method described by Breed and Brew (6) but due to the tendency of the leucocytes to clump the results were not satisfactory.

In determining lactose, the quantity of milk was freed from protein by precipitation with phosphotungstic acid, as recommended by Meyers (6), and the supernatant tested quantitatively with Benedict's solution. Ferric chloride was used for the determination of lactic acid.

The total quantity of material reacting colorimetrically by the procedure of Greenburg (7) for the estimation of proteins was found as follows. The acid milk was first neutralized with normal sodium hydroxide and then centrifuged to eliminate the fat and leucocytes. One cc. of supernatant milk was added to 5 cc. normal sodium hydroxide and after standing for an interval 14 cc. of sodium chloride was added and thoroughly mixed. The mixture was filtered through filter paper and the filtrate tested. The total color reacting substances and those not precipitated by 5 per cent trichloroacetic acid were noted. The whey proteins reacting colorimetrically were determined by the method (8) previously described.

The results with regard to pH, number of leucocytes, and bacterial estimation in 6 spontaneous cases are recorded in table I. In addition the results of the examination of samples taken after an interval of 8 hours are given. The analyses for carbohydrate, proteins and lactic acid are given in table II.

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TABLE 1

The pH, cytological and bacteriological findings in 6 spontaneous cases

ORIGINAL SAMPLES						SAMPLES DRAWN 8 HOURS LATER					
NUMBER	pH	Leucocytes	Colonies of non-hemolytic streptococci per cc. (expressed in millions) dilutions: 10^{-7}	10^{-6}	10^{-5}	pH	Leucocytes	Colonies of non-hemolytic streptococci per cc. (expressed in millions) dilutions: 10^{-7}	10^{-6}	10^{-5}	10^{-4}
5217	5.0	Innumerable (clumped)	160	95	89.6	7.2	11 millions	0	0	0.3	0.20
W.97 R.H.	4.8	"	300	307	220.4	7.2	Innumerable	0	0	0	0.06
L. H.	5.6	"	130	166	141.0	7.0	"	0	0	0	0.05
W. 16	5.0	"	160	384	230.4	6.8	"	0	0	0.4	0.10
5409	4.5	"	320	269	243.2	6.6	24.6 million	0	0	0.6	
P 40	4.8	"	360	323	268.8	6.0	Innumerable	80	98	121.6	
X 7554	5.0	"	20	45	64.0	7.2	"	0	0	0	0.03

TABLE 2

The carbohydrate, protein, and lactic acid findings in 6 spontaneous findings

	ORIGINAL SAMPLES							SAMPLES DRAWN 8 HOURS LATER						
NUMBER	pH	Percentage of carbohydrate	Lactic acid	Mgs. color reacting substance per cc. milk		Mgs. color reacting substance per cc. whey		pH	Percentage of carbohydrate	Lactic acid	Mgs. color reacting substance per cc. milk		Mgs. color reacting substance per cc. whey	
				Total	Not ppt. by 5% trichloracetic	Total	Not ppt. by 5% trichloracetic				Total	Not ppt. by 5% trichloracetic	Total	Not ppt. by 5% trichloracetic
5217 L.F.	5.0	1.3	Strong	43.6	11.2	39.1	5.5	7.2						
W.97 R.H.	4.8	1.4	"	50.0	13.1	28.2	8.0	7.2	2.0	Moderate				
W.97 L.H.	5.6	2.7	"	54.8	14.6	26.4	8.2	7.0	2.2	Weak				
W.16	5.0	1.4	"	64.0	20.0	32.3	10.9	6.8	1.4	Weak	56.7	11.5	29.2	6.9
5409	4.5	1.3	"	44.3	17.3	29.0	14.3	6.6		Negative	60.0	13.5	30.0	8.1
P.40	4.8	To little to detect in 13 cc.	"	64.0	11.2	38.1	7.0	6.0	To little to detect in 15 cc.	Moderate	60.0	9.1	30.4	5.1
7554	5.0		"	47.0	17.0	31.5	10.9	7.2	0.9	Weak	42.0	11.2	22.6	6.5

The original samples were not only acid but the results recorded in Tables I and II indicate that they had other characteristics in common; the large numbers of clumped leucocytes, and enormous numbers of viable streptococci, in every instance the plates with 1 cc. of 10^{-7} milk dilutions developing from 2 to 37 colonies. In all cases, comprising 3 previously reported and the 6 included in Tables I and II, the organisms were the usual non-hemolytic bovine streptococci. All contained relatively little carbohydrate and all gave a strong color when tested with ferric chloride for lactic acid. In addition all had relatively high color reacting substances in the whole milk and in the whey fraction.

In every instance it was possible to obtain milk for re-examination after an interval of 8 hours. The principal differences in the two samples are indicated in Tables I and II. In the chemical constituents there were relatively slight differences, except in the intensity of the reactions for lactic acid, which were much stronger when the milk was distinctly acid. The differences in the proportion of material not precipitated by 5 per cent trichloracetic acid in the first and second samples are sufficiently sharp to indicate that during the first few hours when the milk is acid protein is broken down to a greater extent than during the next few hours. In the main two radical differences are noted between the primary and later samples. The shift in reaction from definitely acid to more alkaline occurs throughout the series. It is true that some become more alkaline than others but all are distinctly less acid in the milk drawn 8 hours after the first specimen. More remarkable is the fact that there is a sharp decline in the number of streptococci. The decline is in a large measure proportional to the shift in pH toward the neutral point. W. 97–R. H. affords an example; here the original sample was pH 4.8 and the organisms numbered about 300 millions per cc. When the milk became alkaline (pH 7.2) the number of streptococci fell to 60,000 per cc. P. 40 is a less striking example; here the first milk obtained had a pH of 4.8 and the count indicated about 360 million streptococci per cc.; later the pH shifted to 6.0 and the number of organisms declined sharply but the 10^{-7} dilution still contained viable streptococci.

In general there is a further correlation between numbers of streptococci per cc. and the degree of acidity; thus in the three samples where the pH is greater than 5.0, the number of streptococci ranges about 300 million per cc., whereas where the pH is between 5.0 and 5.6 the bacterial population is about half or less.

Substantiating evidence that the acidity is definitely correlated with bacterial population can be shown by the data given in table III which sets forth the pH and numbers of streptococci in various portions of the milk drawn during the same milking.

The facts recorded in Table III bear out the data in Table I, *i.e.*, that bacterial population influences the acidity. In the milk from No. 5409 all portions were sharply acid and all contained streptococci in enormous numbers. In the other instances, maximum acidity and the greatest number of bacteria occurred in the fore milk. The middle and last portions of the milk were less acid and the bacterial population was less.

If the phenomenon of acidity in milk within the udder is dependent upon the overwhelming growth of streptococci capable of attacking lactose and thus producing lactic acid, it should be possible by seeding milk with such streptococci to produce the same effect. It must be borne in mind that under conditions where the phenomenon occurs, the organism is required to produce maximum acidity in 8 hours since in the herd from which this material was obtained it is the practice to milk at 8 hour intervals. It is presumed then, that under the natural conditions sufficient growth takes place within this time to change the pH of the milk from 6.6 to 4.5 to 5.0. Experiments dealing with acid production by the non-hemolytic mastitis streptococcus, as indicated by pH under various conditions, bear out this contention.

TABLE 3

Acidity and bacterial populations of the milk from spontaneous infections

NUMBER	FRACTION	pH	STREPTOCOCCI PER 1 CC. (EXPRESSED IN MILLIONS)		
			Dilutions: 10^{-3}	10^{-5}	10^{-6}
P. 40	Fore Milk	4.8	360	323	268.8
	Middle "	5.5	110	217	179.2
	Last "	6.3	170	145	134.4
5409	Fore Milk	4.5	320	269	243.2
	Middle "	4.8	450	325	192.0
	Last "	5.4	290	155	234.0
W. 97	Fore Milk	4.8	300	307	220.4
	Middle "	6.0	80	73	66.5
	Last "	6.0	100	41	51.2

It was first found that veal infusion broth (pH 7.4) containing 2 per cent lactose, when seeded with broth culture of non-hemolytic mastitis streptococci, became acid (pH 4.6) after 8 hours of incubation. Analysis showed that about 0.4 per cent of carbohydrate had been utilized. The bacterial population had increased during the incubation period from 200 thousand per cc. to about 170 million per cc.

When milk which had been previously heated to 58° C. for 20 minutes was inoculated with streptococci and incubated for 8 hours, relatively little change in pH occurred. The same was true when 20 per cent. of unheated cow serum was added to milk; in both instances there was a marked increase in bacterial population but relatively slight changes in pH. When

milk was diluted with salt solution and used as a medium growth was scanty and no change in reaction occurred.

Thus it was established that the streptococcus in a simple medium like broth was capable of attacking sufficient carbohydrate to sharply change the pH, but bacterial growth within the 8 hour period had little influence on the pH in more complex media like milk or milk and blood serum. The acidity of the milk found in the natural infection cannot be ascribed to carbohydrate utilization by the streptococcus in normal milk or mixtures of normal milk and normal blood serum.

It was possible, however, to reproduce the phenomenon when milk from the spontaneous disease was employed as a basic medium, as shown in Table IV.

Experiment.—Acid milk from W. 97 was neutralized with normal sodium hydroxide, and heated to 58° C. for 20 minutes to kill the native streptococci. Milk from a normal cow was also heated to 58° C. for 20 minutes. All tubes were seeded with about 40,000 streptococci per cc. and tested after 8 hours incubation. The results are given in table IV.

The evidence given in table IV is in accordance with the findings in the spontaneous disease. By employing the neutralized abnormal secretion for culture medium it was found that the streptococcus was enabled to produce sufficient acid to bring the pH to 4.6 within 8 hours. The effect of growth

TABLE 4

Effect of bacterial growth on the reaction of normal and abnormal milk

TUBE NUMBER	CONTENT		pH AFTER 8 HOURS	BACTERIAL CONTENT AFTER 8 HOURS (EXPRESSED IN MILLIONS PER CC.)		
				10^{-2}	10^{-4}	10^{-6}
1	5 cc. abnormal milk		4.6			
2	5 cc. abnormal milk + 0.5 cc. 10% lactose		4.6	0	20	20.6
	Abnormal Milk cc.	Normal Milk cc.				
(a)	0.25	4.75	5.6			
(b)	0.5	4.5	5.4			
3 (c)	1.0	4.0	5.0	0	24	15.2
(d)	2.0	3.0	4.8			
(e)	3.0	2.0	4.6			
(f)	4.0	1.0	4.6			
	NaCl Sol. cc.	Normal Milk cc.				
(a)	0.0	5.0	6.0	0	4	5.0
(b)	1.0	4.0	6.2			
4 (c)	2.0	3.0	6.2			
(d)	3.0	2.0	6.4			

is well illustrated in the third series where various proportions of normal and abnormal milk are employed. The effect on pH after 8 hours incubation appears proportional to the quantity of abnormal milk in the mixture. Thus the effect is readily noted when the abnormal milk reaches 20 per cent of the total. The acidity is proportionally higher where greater quantities are used. That the results cannot be ascribed to dilution of normal milk by liquids such as salt solution is also true, since the more dilute the milk the less change in reaction. When similar experiments were done with other strains of non-hemolytic mastitis streptococci and 1,000 and 2,000 streptococci per cc. employed for inoculation, the same results were obtained. Maximum growth and acidity were always found in the abnormal milk and in the tubes containing 20 per cent or more of abnormal milk.

DISCUSSION

In general the components of the milk and bacterial population are similar in the cases of mastitis characterized by acid milk. The total color reacting substances considered as proteins or their split products are increased and to a large measure the increase is comparable to the increase of whey proteins. The secretion is further characterized by the relatively large proportion (25 per cent) of the total color reacting substances which are not precipitated by 5 per cent trichloracetic acid and appears to indicate a relatively active breaking down of tissue elements. The inference is strengthened when the leucocytes contained in the secretion are examined microscopically. The bulk stain badly and are distorted. The shift in the reaction of secretion toward that of neutrality fails to markedly change the composition of the milk but larger quantities of material are precipitated by 5 per cent trichloracetic acid and the leucocytes on microscopic examination are not distorted.

The quantity of lactose secreted during the attack is in all cases small and infers that there is severe involvement of the secretory structures.

The enormous numbers of streptococci found in the milk when it is most acid are significant. The findings in general indicate that the more pronounced the acidity the greater the number of organisms. It seemed reasonable that the phenomenon could be explained on the basis of carbohydrate fermentation by the masses of streptococci, especially as in every instance where the reaction of the milk at the next milking was found to approach more nearly that of the normal or the slightly alkaline secretion usually found in mastitis the bacterial population showed a marked decline. The experiments, however, indicate that comparable acidity cannot be produced in normal milk inoculated with non-hemolytic mastitis streptococci during an 8 hour incubation period. Normal milk then contains, in addition to bacterial inhibitory substances, sufficient buffer to mask the acidity as indicated by pH. If however, the acid milk from the spontaneous disease is first neutralized with sodium hydroxide, mixed in sufficient quan-

tities with normal milk, and inoculated with streptococci, the acidity, as indicated by pH, approaches that found in the spontaneous condition. The clearest results are obtained when only the neutralized acid milk serves as the culture medium. Here the organism may readily attack the carbohydrate and reach its limiting hydrogen ion concentration within the 8 hour period.

It seems to us, then, that there is a threefold explanation of the phenomenon; the milk within the udder is relatively poor in buffer, it contains sufficient protein split products to be comparable with broth as a culture medium, and the organisms are enabled to grow at a maximum rate and attack carbohydrate sufficiently to definitely change the pH.

The shift toward the neutral point at the next succeeding milking may be explained by the fact that the udder has been drained of acid milk, thus greatly diminishing the number of organisms as well as relieving the local pressure. As the circulatory functions improve and permit the passage of larger quantities of blood base the buffer value of the secretion is increased. The character of the medium is changed and the streptococci multiply less rapidly.

SUMMARY

The findings in definitely acid milk from acute udder disease are discussed. In all instances the condition was characterized by the large numbers of non-hemolytic streptococci and leucocytes in the secretion. Lactic acid was always present. The condition is correlated with the enormous growth of carbohydrate attacking streptococci within the udder in a relatively poorly buffered altered milk medium.

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INFLUENCE OF DIFFERENT STARTERS ON THE QUALITY OF CHEDDAR CHEESE

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INTRODUCTION

The use of a starter in the manufacture of cheddar cheese is an accepted practice resorted to for the rapid development of lactic acid. Lactic acid is necessary to properly coagulate the milk with rennet, to restrict the growth of undesirable organisms, and to properly control the moisture content and texture of the finished cheese. Production of lactic acid is not the only function of a starter, for most investigators believe that the organisms present in the starter are extremely important in breaking down the insoluble casein compounds and in producing a characteristic flavor. Just what organisms are responsible for all the flavor production in cheese is not known, but in butter those producing volatile acid are known to be very important and desirable.

Hammer and Bailey (1) have shown that commercial starters are composed of more than one organism. Two of the organisms present in commercial starter, and important because of their ability to produce large amounts of volatile acid, are *Streptococcus citrovorus* Hammer and *Streptococcus paracitrovorus* Hammer. Hucker and Marquardt (2) secured a desirable effect upon the flavor of cheddar cheese when using *Streptococcus paracitrovorus* alone or in conjunction with commercial starters. The same investigators found that *Streptococcus citrovorus* appeared to have no effect upon the flavor of cheddar cheese.

The data in this paper are presented as a contribution to the limited knowledge on the quality of cheddar cheese as affected by the use of various starters and cultures.

METHODS

The milk used was produced by the university dairy herd consisting of Holsteins and Jerseys. The average butterfat content of the milk was approximately 3.6 per cent. The milk was dumped into a large coil pasteurizer, thoroughly mixed, and analyzed for fat, titratable acidity, and bacterial content. Portions of 200 pounds each were used for making

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cheese. The standardized procedure for manufacturing cheddar cheese was followed, the methods in all batches being as nearly alike as possible. All comparisons were not strictly confined to split batches, but the quality of the milk used was quite uniform from day to day so that the results should be comparable. Cheese was made from both raw and pasteurized milk. The following methods were used with pasteurized milk; namely, the same procedure as used for raw milk, and the procedure recommended by Sammis and Bruhn (3).

The pasteurized milk was heated to 142° to 145° F., held for 30 minutes, and then cooled to 88° F. The amount of starters and cultures used varied with the type of culture. Twelve cc. of rennet extract were added per 100 pounds of milk. All cheese was weighed and paraffined when seven days old. Each batch of cheese was scored for flavor, body and texture, and color at 6, 12, and 18 weeks of age. The scoring was done by two judges scoring independently of one another. The score card used was that approved by the Western Division of the American Dairy Science association which did not permit any criticism of flavor when the score was 37 or above.

Moisture content of the cheese was determined at the time of dressing and at 6, 12, and 18 weeks of age by the method recommended by the A. O. A. C. The storage temperature was maintained between 48° and 59° F.

Starters and Cultures Used

The starters and cultures used were:

Commercial starters
- Ericsson
- Ames No. 122

Cultures
- *Streptococcus lactis*
- *Streptococcus citrovorus*
- *Streptococcus paracitrovorus*

The Ericsson commercial starter was secured from the Elov Ericsson company, St. Paul, Minnesota. Ames starter No. 122 and the pure cultures of *Streptococcus citrovorus* and *Streptococcus paracitrovorus* were secured from Dr. B. W. Hammer, Iowa State college, Ames, Iowa. The pure culture of *Streptococcus lactis* was isolated in the University of Idaho laboratory from Ames starter No. 122. All pure culture starters were examined by the direct microscopic and standard plate methods at the time of using for both purity and viability.

EXPERIMENTAL

Table 1 shows the flavor, and body and texture scores at 6, 12, and 18 weeks of age and the moisture content at dressing and 18 weeks of age of

TABLE 1

Influence on flavor, body and texture, and moisture content

STARTER USED	AGE AT SCORING WEEKS	RAW				PASTEURIZED				PASTEURIZED WISCONSIN			
		NO. BATCHES	Flavor	Body and Texture	Moisture at Dressing	Moisture at 18 Weeks	NO. BATCHES	Flavor	Body and Texture	Moisture at Dressing	Moisture at 18 Weeks	NO. BATCHES	Flavor	Body and Texture	Moisture at Dressing	Moisture at 18 Weeks
Ericsson	6		36.40	29.05				36.90	29.55				38.00	29.50		
1 to 2 per cent	12	5	36.75	28.95	37.94	35.76	5	38.65	29.55	39.04	35.70	1	36.50	29.50	38.00	35.10
	18		36.60	28.90				37.20	29.10				38.00	29.25		
	6		36.33	29.50				36.25	29.25							
Ames	12	3	38.33	28.67	38.93	36.73	3	37.66	29.00	40.20	38.87					
1 to 2 per cent	18		36.33	28.50				38.00	29.50							
	6		36.17	29.33				37.17	29.67				36.00	29.50		
S. lactis	12	3	38.00	29.67	37.20	36.26	6	36.35	29.40	37.31	34.60	3	37.00	29.33	37.73	35.66
1 to 2 per cent	18		37.33	29.58				36.37	29.67				37.45	29.58		
	6		36.58	28.84				34.80	29.10				36.68	29.25		
S. paracitrovorus	12	3	33.00	28.50	39.00	36.33	5	34.20	28.20	40.15	36.76	3	37.33	28.66	40.15	37.66
Less than 4%	18		34.50	28.17				36.50	29.10				37.50	29.17		
	6							33.00	29.00							
S. paracitrovorus	12						3	33.33	29.16	40.03	36.26					
More than 4%	18							33.92	28.83							
	6		35.50	28.50				34.00	28.00				35.50	28.33		
S. citrovorus	12	3	33.50	28.17	38.36	35.83	1	32.25	27.25	42.30	40.00	3	30.00	28.50	38.53	33.60
Less than 4%	18		34.17	28.17				34.00	29.00				33.88	28.33		
	6							34.87	28.62							
S. citrovorus	12						4	34.94	29.25	38.47	34.40					
More than 4%	18							35.75	29.19							
	6							37.33	29.66							
S. lactis, 1 to 2%	12						3	36.00	29.66	37.23	33.80					
S. paracitrovorus, 1%	18							37.50	29.50							
	6							36.83	29.16							
S. lactis, 1 to 2%	12						3	36.33	29.50	36.23	34.66					
S. citrovorus, 1%	18							36.16	29.50							
	6							36.00	29.00							
S. lactis, 1%	12						2	34.00	29.00	37.95	35.30					
S. paracitrovorus, 1%	18							35.00	29.25							
S. citrovorus, 1%																

all cheese made by the three different methods using the various starters and cultures.

Ericsson Starter

The Ericsson starter used was one which is used by many cheese makers. While no definite study was made of the type of organisms present in this starter, it may safely be assumed that such common types as *Streptococcus lactis, Streptococcus cremoris,* and perhaps *Streptococcus citrovorus* or *Streptococcus paracitrovorus* were present. Cheese made from milk inoculated with this starter had a satisfactory flavor and good body and texture when either raw or pasteurized milk was used. At any stage of curing, the flavor, as well as the body and texture, of the cheese made from pasteurized milk was slightly better than that of the cheese made from raw milk. The moisture content of the cheese made from pasteurized milk was slightly higher, but the loss of moisture slightly greater than with the cheese made from raw milk so that the moisture content after 18 weeks of curing was approximately the same.

Ames Starter

The Ames starter contained *Streptococcus lactis, Streptococcus citrovorus,* and *Streptococcus paracitrovorus.* The proportion in which these organisms were present in the starter was not definitely determined. Cheese made with the Ames starter had a satisfactory flavor and good body and texture when either raw or pasteurized milk was used. Cheese made with this starter was equal in flavor and body and texture to the cheese made with the Ericsson starter, whether raw or pasteurized milk was used. Pasteurization of the milk slightly improved both the flavor and body and texture of the cheese, especially at the age of 18 weeks. As with the Ericsson starter, the moisture content and loss of moisture was higher in the pasteurized milk cheese, and it was highest when the Ames starter was used, probably because of the lower acidity produced by this starter at the time of drawing of whey. The slow development of acidity by this starter may at times have been responsible for an inferior texture in the cheese. Differences in the time necessary to manufacture cheese with the Ericsson and Ames starters were too small to be important.

Streptococcus lactis

The strain of *Streptococcus lactis* used gave satisfactory results as shown by the flavor and body and texture scores when either raw or pasteurized milk was used. Although the flavor of the cheese after curing was slightly inferior when this culture was used as a starter than when either the Ericsson or Ames starter was used, the body and texture scores of the cheese were the highest for any starter used and the moisture content was

the lowest, both at 12 and 18 weeks of age. Here again the loss of moisture in the pasteurized milk cheese was greater than in the raw milk cheese. All steps in the manufacturing process proceeded normally.

Steptococcus paracitrovorus

Streptococcus paracitrovorus produces relatively large amounts of volatile acid and according to Hucker and Marquardt (2) has a desirable effect upon the flavor of cheese when used either alone or in conjunction with commercial starters. In these trials, however, the use of *Streptococcus paracitrovorus* in raw milk resulted in cheese with a characteristic bitter flavor and open body and texture. However, when pasteurized milk was used a more pleasant flavor was produced, particularly noticeable when the cheese was 18 weeks old. The body and texture was also better in the pasteurized milk cheese than in the raw milk cheese, but it was somewhat inferior to the texture of the cheese made with the Ericsson, Ames, and *Streptococcus lactis* starters. The best cheese made with this culture was made from pasteurized milk using the Wisconsin method. Probably the addition of hydrochloric acid overcame, at least partially, the outstanding disadvantage of using this culture, which is its inability to produce acids, either quickly or in large amounts. *Streptococcus paracitrovorus* when used in amounts exceeding 4 per cent, by volume, in pasteurized milk produced cheese with a decidedly bitter flavor. The moisture content of all cheese made with this culture was relatively high. The manufacturing process was slightly prolonged because of the slowness of the development of acidity.

Streptococcus citrovorus

Streptococcus citrovorus produces a small amount of acid and a relatively large amount of volatile acid. According to Hucker and Marquardt (2) *Streptococcus citrovorus* when used alone or in combination with commercial starters has no effect upon the flavor of cheddar cheese, but in this work the use of *Streptococcus citrovorus* resulted in a poor quality cheese. Regardless of the kind of milk (raw or pasteurized), method of manufacture, or amount of culture used (up to 10 per cent), unsatisfactory cheese was obtained. The flavor was usually distinctly bitter and the body decidedly open. As with *Streptococcus paracitrovorus,* the time necessary to make cheese when using this culture was slightly longer, due to the slow development of acidity.

Mixed Cultures

Since the use of *Streptococcus citrovorus* and *Streptococcus paracitrovorus* alone produced no successful results except when *Streptococcus paracitrovorus* was used in pasteurized milk acidified with hydrochloric acid, it was thought that the mixture of either of these organisms with

Streptococcus lactis might produce better cheese. A mixture of 2 per cent, by volume, of *Streptococcus lactis* with 1 per cent of either *Streptococcus citrovorus* or *Streptococcus paracitrovorus* produced a good flavored and textured cheese with pasteurized milk. The mixture of 2 per cent of *Streptococcus lactis* and 1 per cent *Streptococcus paracitrovorus* produced the better flavored cheese of the two mixtures. A mixture of 1 per cent, by volume, of *Streptococcus lactis*, 1 per cent *Streptococcus paracitrovorus*, and 1 per cent *Streptococcus citrovorus* was tried with two batches and a fair quality cheese resulted. All of the mixtures used produced better flavored cheese with pasteurized milk than either *Streptococcus paracitrovorus* or *Streptococcus citrovorus* alone. The number of trials made with the mixed cultures of *Streptococcus lactis*, *Streptococcus paracitrovorus*, and *Streptococcus citrovorus* was limited and consequently definite conclusions cannot be made.

Fat Losses in Whey and Yield

There was little difference in the fat losses in the whey among the different starters and cultures used. Fat losses in the whey were slightly greater when using raw milk than when using pasteurized milk. There was no appreciable difference in the yield when using the different starters and cultures. Pasteurized milk gave a slightly higher yield than raw milk, probably due to the higher moisture content of the pasteurized milk cheese and the lower losses of fat in the whey.

SUMMARY AND CONCLUSIONS

Cheddar cheese was made from raw and pasteurized milk inoculated with the following starters and cultures: Ericsson, Ames 122, *Streptococcus lactis*, *Streptococcus paracitrovorus* Hammer, and *Streptococcus citrovorus* Hammer.

Use of the Ericsson starter resulted in cheese with a satisfactory flavor and body and texture with either raw or pasteurized milk.

Cheese made with the Ames starter, whether using raw or pasteurized milk, was equal in flavor and body and texture to the cheese made with the Ericsson starter.

The flavor of cheese made with the *Streptococcus lactis* culture was almost equal to that of cheese made with the Ericsson and Ames starters, but the body and texture was superior to that of the cheese made with these two starters.

The use of *Streptococcus paracitrovorus* in amounts of less than 4 per cent, by volume, in raw milk resulted in cheese with a characteristic, bitter flavor and open body and texture. However, when pasteurized milk was used a more desirable cheese was made, particularly when the milk was acidified with hydrochloric acid. This culture, when used in amounts from

4 to 10 per cent in pasteurized milk, resulted in cheese having a decidedly bitter flavor.

Streptococcus citrovorus when used in amounts ranging from 1 to 10 per cent, by volume, produced a distinctly bitter flavor and a decidedly open body and texture in cheese.

Cheese made with mixed cultures of *Streptococcus lactis, Streptococcus paracitrovorus,* and *Streptococcus citrovorus* was of fair quality. The quality of this cheese was superior to that of cheese made from milk inoculated with pure cultures of *Streptococcus paracitrovorus* or *Streptococcus citrovorus,* but inferior to the quality of cheese made from milk inoculated with either a pure culture of *Streptococcus lactis* or the Ericsson and Ames starters. To justify definite conclusions additional data on the use of these mixtures should be obtained.

Cheese made from pasteurized milk was equal in flavor and superior in body and texture to the cheese made from raw milk.

Cheese made from pasteurized milk when fresh had a higher moisture content than cheese made from raw milk. The loss of moisture through a period of 18 weeks' storage, however, was usually greater in the cheese made from pasteurized milk than in cheese made from raw milk.

Slightly higher yields of cheese were obtained from pasteurized milk than from raw milk.

Fat losses in the whey were slightly lower in cheese made from pasteurized milk than in cheese made from raw milk.

There were no appreciable differences in yields or in fat losses in the whey of cheese made with the different starters and cultures.

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EXPERIMENTAL INITIATION OF MILK SECRETION IN THE ALBINO RAT*

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In a recent paper a study of the causes of the normal development of the mammary glands of the albino rat was reported by Turner and Schultze (12). It was found that the estrogenic hormone was capable of inducing the mammary duct development that normally occurs in the female at the approach of puberty. After puberty is reached and the growth of the gland is under the influence of the short recurring estrus cycles characteristic of the rat, slight lobule proliferation was found along the branches of the ducts. Inasmuch as slight lobule proliferation was induced by large doses (10 to 15 rat units) of the estrogenic hormone, this growth in the virgin adult rat was assumed to be due to this hormone, although the presence of corpora lutea in the ovaries during the cycle may influence the type of growth to some extent.

It was reported also that the estrogenic hormone administered simultaneously with a corpus luteum extract induced lobule proliferation considerably greater than that induced by the estrogenic hormone alone; but did not induce the dense hyperplasia of the mammary gland that appears at the fifteenth day of pregnancy. This observation is explained by other experimental work which will be reported later in this paper.

The failure to obtain milk secretion in the albino rat from an extract of sheeps' pituitary was also reported.

Recent studies show that the stimulus to the initiation of milk secretion is obtained from a hormone produced by the anterior pituitary. Stricker and Grüter (10) obtained milk secretion in pseudo-pregnant ovariectomized rabbits by an extract of the anterior pituitary. Corner (1) obtained lactations in immature ovariectomized females with alkaline extracts of sheeps' pituitary prepared by Parke, Davis and Company. Nelson and Pfiffner (7) produced lactation in rabbits, guinea pigs, and rats with the alkaline extract of sheeps' pituitary obtained from Parke, Davis and Company. They emphasize the necessity of sensitizing the mammary gland by action of a corpus luteum hormone before the stimulus of the pituitary hormone is effective.

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Aqueous extracts of the anterior pituitary and fresh pituitary implants when injected into normal female rats was observed by Evans and Simpson (2) to cause mammary gland growth and milk secretion, but failed to perform this function in spayed females.

In this laboratory an alkaline extract of sheep's pituitary has been found effective in bringing mature spayed female rabbits into lactation without preliminary treatment (Turner and Gardner, 11). However, these same extracts have been ineffective in bringing about milk secretion in mature female rats (both normal and spayed) whose glands had been developed by previous periods of pregnancy and were in various stages of involution. Experiments were begun, therefore, to determine if preliminary treatment was necessary to condition the mammary glands before the pituitary extract was effective in producing secretory activity.

Reports have been made also by several investigators indicating a relationship between the estrogenic hormone and the initiation of milk secretion. Steinach, Dohrn, Schoeller, Hohlweg and Faure (9) obtained milk secretion in castrate guinea pigs after continued injection (7–8 weeks) of the estrogenic hormone. Laqueur, Borchardt, Dingemanse and DeJongh (5) injected castrate female guinea pigs with the estrogenic hormone for 14 days and obtained secretion. Fellner (3) from his results obtained with rabbits, concluded that the estrogenic hormone prepares the gland for lactation but lactation does not result until the injections of the hormone are discontinued.

Procedure

The albino rat was used in all the experiments conducted. All injections of extracts and implants were given subcutaneously. Except where otherwise stated the animal was killed and a gland removed one day following the last injection. In some animals control glands were removed prior to the experiment. Otherwise, the normal development as determined previously, served as the method of comparison for interpretation of results. In several experiments, glands were removed from the animal during the progress of the experiment to determine the progressive changes that had occurred. Only a representative section of the gland was removed, since a knowledge of the type of development induced rather than the extent of gland area was desired. Total mounts of the removed glands were made for macroscopic examination following the same technique described previously (Turner and Schultze, 12). In some instances histological preparations were made.

The source of the estrogenic hormone used was an oily extract of pregnant cows' urine. As this extract has been shown to induce growth of the mammary glands similar to that induced by the crystalline estrogenic hormones, theelin and theelol (Turner, Frank, Gardner, Schultze, and Gomez,

13) the active fraction present in the oily extract of pregnant cows' urine will henceforth be called theelin.

The corpus luteum extract used was prepared in this laboratory from fresh sows' corpora by a method described by Fevold, Hisaw, and Leonard (4). Of the three fractions isolated by these workers, only the fraction called corporin, which induces "progestational proliferation" of the uterus was isolated and tested in this laboratory. The alkaline extract of sheeps' pituitary was prepared by a method described by Turner and Gardner (11). Because of its true galactogogic effect this hormone will be called "galactin."

The fresh implants of whole pituitaries were obtained from both male and female rats.

Experimental Work

Continued study of the causes of the growth of the mammary gland of the albino rat has confirmed past observations and indicated an explanation for certain phases of growth hitherto not very well understood.

In seeking an explanation for the continued duct development occurring in the male and female albino rat, ovaries from 20 day old and 32 day old females were sectioned. The sections showed in both cases numerous immature follicles filled with follicular fluid. Thus the source of the theelin causing the duct development in the immature female may be assigned to the secretion of follicular fluid in the immature ovary.

Extracts of the urine and feces of four groups of male rats were made and tested for the presence of theelin. The youngest group was 53 days of age, while the oldest group was mature males. An average of about 800 gms. of excreta was extracted. Two-thirds of the extracted oil from this amount of excreta in three of the groups caused a positive estrus vaginal smear in spayed female rats. The large quantity of extracted oil resulting from the group 3½ months of age made it impossible for more than one-fourth of the total extract to be injected in a one day period and the vaginal smear obtained was not completely cornified although cornified cells were present. From this evidence it is believed that some organ in the male produces the theelin causing the characteristic duct development in the male rat. That the testes may elaborate this hormone is indicated by work of Maino and Frattini (6). These investigators report the extraction of a crystalline substance from testes which caused typical estrum when injected into spayed female rats.

It was stated above that the growth induced by the simultaneous administration of theelin and corporin induced lobule proliferation greater than that induced by theelin alone, but did not induce the dense hypertrophy of the mammary gland that is characteristic of the later stages of pregnancy. In this connection, the type of gland growth induced during

pseudo-pregnancy was studied. During pseudo-pregnancy in the rat no secretory activity is stimulated in the gland, but both ovarian hormones, theelin and corporin, may be assumed to be active for a relatively long period (13–16 days). The gland growth resulting from the condition of pseudo-pregnancy is not nearly so dense as that present in a female pregnant for 15 days. There is formed, however, lobule development apparently comparable to the amount present in the gland of a female pregnant for 13 days. As there is no gradual upbuilding of secretory activity going on during pseudo-pregnancy and from the fact that in the absence of the ovaries the lactation-promoting hormone of the anterior pituitary does not cause any gland growth, it is thought that full growth of the mammary gland exclusive of secretory activity is induced during pseudo-pregnancy. Roberts (8) similarly concluded from his study of the mammary gland of the albino rat during pregnancy that from the fact that there is an absence of cell division and no increase in the number of cells about the alveoli, the gland had reached its maximum growth so far as the number of cells was concerned by the thirteenth day of pregnancy. From a cytological study of the mammary gland of the albino rat, Weatherford (14) observed that the mammary gland of the pregnant rat shows the greatest growth early in the period of gestation and that evidences of cellular proliferation are extremely rare in the second half of that period. The gland growth induced experimentally by theelin and corporin together is apparently similar to that induced by pseudo-pregnancy. It is thought, therefore, that the complete growth of the gland—the duct system and alveoli—is induced by these two hormones; but since no milk secretion is stimulated, the great hypertrophy characteristic of the gland during advanced pregnancy does not occur.

In view of the apparent relationship between theelin and milk secretion, a series of experiments were begun to determine if varying amounts of theelin injected into female rats both normal and spayed, would induce milk secretion directly, or if milk secretion would result on discontinuation of the administration of this hormone; or if this hormone so prepared the gland that it would be capable of being stimulated to secretory activity by the alkaline extract of the sheeps' pituitary.

A group of mature virgin females were spayed. Two were treated with one rat unit of theelin for 18 days. The gland removed after this treatment showed the characteristic growth induced by small amounts of theelin, namely, an extensive duct system with a few small lobules. No evidence of secretion was apparent. One of these females after the theelin treatment received an additional treatment with 0.8 c.c. of galactin daily for 9 days; the others were given no further injections. Glands from both animals were removed on the tenth day following the discontinuance of theelin injection. Both glands, one from the individual receiving the addi-

tional treatment with galactin, and the other removed ten days after the discontinuance of theelin, showed no apparent evidence of secretory activity.

Two other spayed females treated similarly to those just described with the exception that 2 rat units of theelin daily were injected for 18 days. No secretory activity was induced in these individual glands. Two other similar spayed females treated in the same manner except that 15 rat units of theelin were injected daily showed glands that had extremely large club-shaped end buds, but no milk secretion was evident.

To further investigate the influence of theelin on the initiation of milk secretion, mature females that had previously had young and therefore had mature but involuted mammary glands were used. Two were spayed and two left normal. Each animal was treated with 50 rat units of theelin daily for 20 days. In each case there was very little or no apparent change in the glands after the theelin treatment and in the control gland removed before the experiment was started. Apparently theelin is incapable of rejuvenating the lobules from their inactive state either in normal or spayed females. An additional treatment following this theelin treatment, of 0.3 c.c. of galactin for 10 days did not induce any distension of the lobules or secretory activity of the involuted mammary glands of either normal or spayed females.

Two mature spayed females that had previously borne young and whose glands were in an involuted state were treated with 5 rat units of theelin plus 0.3 c.c. of galactin for 30 days. The glands after such treatment apparently were not significantly different from the control glands removed before the experiment. No evidence of milk secretion could be observed.

The results of these experiments indicate that theelin is incapable of inducing, either directly or indirectly through the animal's own pituitary, the initiation of milk secretion in the albino rat. Neither does it seem to be capable of conditioning the gland so that the pituitary extract galactin is effective in initiating secretion.

It was thought possible that negative results obtained in these experiments might be due to the relative instability of the hormone in the extract. For this reason fresh pituitary implants were used to determine their effects on mammary gland development and the initiation of milk secretion.

A normal multipara female was given a fresh pituitary implant daily for six days, the first implant being given 11 days after the weaning of her litter. The pituitaries implanted were obtained from pregnant female rats at the time of (or shortly after) parturition. It was thought that at this stage the pituitaries would be the most potent for the lactation-stimulating effect. The control gland from this female showed the presence of many lobules, but macroscopically the density of the gland was much less than that of a gland in lactation. Involution had not continued to the

state where the gland was similar to that of a virgin female. The gland removed after the pituitary implantations was much denser in appearance when compared to the control. Apparently the alveoli were much more distended in the gland following the treatment. Examination of histologic sections of the glands verified the macroscopic observations. The control gland showed shrunken alveoli with very small lumen, while the gland removed following treatment showed lumen greatly increased in size and filled with fluid. However, the degree of secretory activity induced was not nearly as great as that occurring at the time of parturition. In all probability the length of time of the experiment was too short to bring the gland into a state of complete lactation.

Another normal multiparous female whose mammary glands were considerably involuted was treated with six pituitary implants over a period of 14 days. The pituitaries used were taken from spayed females. A control gland was removed before the experiment and showed a characteristic feathery appearance due to the shrunken lobules. A gland removed on the seventh day after the beginning of the experiment showed considerable distension of the previously shrunken alveoli. A gland removed on the fourteenth day after the beginning of the experiment showed secretory activity and development comparable to that of a gland from a female pregnant for 17 days.

Experiments were begun to determine the effect of pituitary implants on immature females. A normal female 55 days of age was treated with a fresh pituitary implant from male rats daily for 15 days. The gland removed after this treatment showed much lobule development and much distension of the alveoli. The gland was comparable to that of one present in a female pregnant for 15 days.

The pituitary implantations were continued for an additional six days and a gland removed. The gland resulting from the effects of the 21 day period of treatment showed secretory activity apparently as great as that occurring normally at the time of parturition. The alveoli were well distended with milk and the gland was as thick as a gland at the time of parturition. Apparently the pituitary implants in females with ovaries intact are capable of rejuvenating the involuted mammary glands of mature multiparous females to full lactation, and in immature females that normally have only a duct system these pituitary implants are capable of inducing full growth of the gland (an extensive duct system and lobulation); and to bring this developed gland into milk secretion.

With these effects produced by pituitary implants on the normal female albino rat, experiments were begun to determine if like results could be obtained in spayed females with pituitary implants.

Two multiparous females with mammary glands in an involuted state were spayed and treated with a pituitary implant from males every other

day for a period of 30 days. No milk secretion was obtained and the glands remained in an involuted condition.

Immature spayed females treated with pituitary implants for 15 days showed glands that had undergone no significant development as a result of the treatment.

An immature spayed female when treated with two pituitary implants before being spayed to induce pseudo-pregnant growth of the mammary glands and treated after spaying with a pituitary implant daily plus 10 rat units of theelin daily for 10 days did not show glands in which milk secretion had been initiated.

These experiments indicate that in ovariectomized females the fresh pituitary implants are incapable of initiating milk secretion or any growth of the gland in immature females.

From the fact that gland growth and milk secretion was induced by fresh pituitary implants in females with intact ovaries it was assumed that the pituitary implants caused precocious development of the immature ovaries and therefore the liberation of theelin and corporin. This would indicate that the action of theelin and corporin is necessary together with the lactation-promoting hormone to induce milk secretion even when mammary gland growth induced by pseudo-pregnancy is present.

Therefore, a normal immature female rat was treated with two pituitary implants, given at a day's interval apart to induce pseudo-pregnant growth of the gland. Nine days after the first pituitary implant was given she was spayed. Following spaying she received .15 c.c. of the corporin extract daily and a fresh pituitary implant every other day for 10 days. The gland resulting in this spayed female from this treatment showed dense lobule growth and milk secretory activity apparently equal to that present in the gland of a female pregnant for 16 days. Although no theelin was injected with the pituitary implants, the animal came into estrum as shown by the vaginal smear present. Apparently theelin was present in the corpus luteum extract.

The results of this experiment lends support to the assumption that theelin and corporin are necessary before the lactation-stimulating hormone is effective in initiating secretory activity.

Inasmuch as previous experiments with the alkaline extract of sheeps' pituitary alone or in conjunction with theelin failed to produce milk secretion either in normal or spayed females, the results obtained with fresh pituitary implants led to the experiment of trying corporin and theelin with the pituitary extract.

A normal multipara female whose mammary glands were in an involuted state was used. She received 5 rat units of theelin plus 0.1 c.c. of corporin for three days; 5 rat units of theelin plus 0.1 c.c. for two days, and 0.4 c.c. of galactin alone for five days, making a total injection period of

ten days. The gland removed following this treatment showed considerable secretory activity. Although the thickness of the gland was not as great as in a gland at the time of parturition, the alveoli were well distended with secretory products. Apparently the gland was comparable to a gland of a female pregnant for 16 days.

A mature multiparous female whose mammary glands were in a state of involution almost equal to that present in a virgin female was spayed. Following spaying this female was treated with 0.1 c.c. of corporin plus 2.5 rat units of theelin plus 0.4 c.c. of galactin for four days. Following this she received an additional treatment of 0.4 c.c. of galactin daily for four days. A gland removed after this treatment showed distension of the alveoli when compared to the control gland. An additional treatment of 0.4 c.c. of galactin caused no further increase in the secretory activity of the glands.

Evidently the extract of sheeps' pituitary that is able to initiate secretory activity in the rabbit mammary gland is also able to induce the same phenomenon in female rats, provided the growth of the gland has taken place and that corporin and theelin are present.

Discussion

The results obtained as described above indicate that the hormone of the anterior pituitary galactin is capable of initiating milk secretion in the albino rat the same as in the rabbit. The species difference between the rat and rabbit apparently lies in the fact that it is necessary to condition the gland of the albino rat before the stimulus to secretion is able to act.

In the normal growth of the mammary gland and the initiation of secretory activity during pregnancy, it is probable that theelin together with corporin causes the development of the duct system and proliferation of the lobular structures. This development apparently occurs during the first half of pregnancy. During the latter half of pregnancy and while the gland is still under the influence of theelin and corporin, the lactation-stimulating hormone from the anterior pituitary is becoming active and gradually stimulating the cells of the alveoli to secretory function. This is evidenced by the fact that during the last half of pregnancy there is a gradual distension of the alveoli and an accumulation of secretory products within the lumen of the alveoli.

Long continued treatment of normal immature female rats with pituitary implants, as related above, brought about copious milk secretion. The hormones brought into action to influence mammary gland activity with such treatment would appear to simulate those operating during normal pregnancy.

Supposedly the gonad-stimulating and luteinizing hormones of the pituitary implants caused precocious maturity of the ovaries, thereby releasing

the theelin and corporin which bring about growth of the duct system and lobules of the mammary glands. While the influence of these hormones is still in operation the lactation-stimulating fraction of the anterior pituitary brings about a gradual upbuilding of secretory activity in the susceptible gland. If this explanation as to what happens is correct, then the lack of sufficient growth of the gland would account for the failure of pituitary implants or extracts to initiate secretory activity in the spayed immature female.

However, even when mature mammary gland development is present as in multiparous females with involuted glands, the pituitary implants alone are unable to bring the gland of such spayed animals into secretory activity and the pituitary extract is incapable of initiating secretion in either spayed or normal animals. Furthermore when growth of the gland was induced by pseudo-pregnancy in an immature female rat and then spayed, no milk secretion resulted on treatment with pituitary implants. In all females both spayed and normal whose glands were mature but in a dry condition it was found that only by giving a preliminary treatment with theelin and corporin was the alkaline extract of the pituitary able to bring about secretion. It seems, therefore, that a sensitizing influence on the grown mammary gland is necessary before lactation can be brought about in the female albino rat. In one animal this sensitizing effect was brought about by an extract of the corpus luteum without the separate injection of theelin. However, the fact that the animal so treated came into estrum indicates that theelin was present in the corpus luteum extract.

The lack of gonad-stimulating hormones in the alkaline extract of sheeps' pituitary and which are present in fresh pituitary implants would explain the ineffectiveness of the pituitary extract in causing milk secretion in normal females such as was induced by pituitary implants.

The entire absence of mammary gland development when immature spayed female rats were treated with pituitary implants alone and the lack of continued or additional growth in spayed virgin females when treated with the alkaline extract of sheeps' pituitaries indicates that mammary gland growth is not induced directly by action of the pituitary hormones. Only when the ovary is intact and by action indirectly through the ovary is the pituitary able to cause mammary gland growth.

It is noted in the experimental observations that fresh pituitary implants obtained from male rats brought about milk secretion in normal immature females. Thus the lactation-stimulating hormone appears to be present in the male as well as the female pituitary.

Summary

Additional experimental evidence is reported in support of the theory that the growth of the mammary duct system is stimulated by the estro-

genic hormone theelin; that the lobular system is stimulated by theelin and corporin; and that the secretory activity is initiated by a hormone from the pituitary. The presence of numerous follicles in infantile rat ovaries suggests that the estrogenic hormone may be secreted by these ovaries and thus be responsible for the duct development in the immature female.

The positive tests for theelin obtained from extracts of urine and feces of male rats indicate that some organ in the male elaborates the estrogenic hormone which is believed to be responsible for the rather extensive duct development occurring in the male rat gland.

Mammary gland development induced by a pseudo-pregnant condition indicates that full development of the duct system and lobules occurs during the first half of pregnancy.

The estrogenic hormone alone seems incapable of initiating milk secretion or of conditioning the gland so that pituitary extracts can stimulate the gland to secretion.

Milk secretion is induced in normal females by subcutaneous implants of fresh pituitaries from either male or female donors. In spayed females the presence of theelin and corporin was necessary before milk secretion was brought about by pituitary implants.

The extract of sheeps' pituitary was able to induce secretory activity in either spayed or normal females only when theelin and corporin were injected with the galactin.

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THE INFLUENCE OF MEDIA UPON THE YEAST AND MOLD COUNT OF BUTTER

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INTRODUCTION

In the report of the Committee on Suggested Methods for the Microbiological Analysis of Butter (1) four media are given for the estimation of the yeast and mold content of butter. Three of the four suggested media are whey agars prepared in different ways, the fourth a dehydrated malt agar. After publishing this report, suggestions were offered by Grimes (2) and Meyers (3) relative to the use of other media for the estimation of yeasts and molds. The American investigators, Hammer (4), Macy (5), Bouska (6), and Parfitt (7), when determining yeasts and molds have used whey agar while the Canadian investigators, Hood and White (8), have used malt agar. White and Hood (9), (10), in their studies on temperature and hydrogen-ion concentration used Bacto dehydrated malt agar.

OBJECT

Because of the difference of opinion as to the relative merits of various media for the estimation of yeast and mold count in butter it was deemed desirable to make a study of the relative merits of the various media which may be used for yeast and mold count.

PROCEDURE

The experiment was divided into two parts. Part one in which the following media were compared:

1. Whey agar prepared by coagulation with rennet
2. Beef infusion agar plus 1 per cent lactose
3. Standard nutrient agar plus 5 per cent dextrose, suggested by Henrici (11)
4. Wort agar dehydrated, Difco
5. Malt agar dehydrated, Difco
6. Standard nutrient agar
7. Standard nutrient agar plus 1 per cent lactose

Part two in which the following media were compared:

1. Whey agar prepared by coagulation with rennet
2. Whey agar prepared by coagulation with acid
3. Whey agar dehydrated, Difco (batch 1)
4. Whey agar dehydrated, Difco (batch 2)

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5. Standard nutrient agar plus 5 per cent dextrose
6. Wort agar dehydrated, Difco
7. Malt agar dehydrated, Difco

PROCUREMENT OF SAMPLES

Samples of butter were procured at random and upon reaching the laboratory were sampled in the manner suggested by the Committee on Microbiological Analysis of Butter. (1) After sampling the butters were returned to a cold room (40°F.). A second sampling was made after the dilution had been determined.

PRELIMINARY PLATING

To determine the dilution necessary for each sample of butter, a preliminary plating was made using a number of dilutions. Incubation was at 25° C. for 5 days. Plates were counted and results recorded to prepare a suitable dilution in which the seven media were compared. In the first series whey agar coagulated by rennet was used as the preliminary medium, in the second series standard nutrient agar plus 5 per cent of dextrose was used as the preliminary medium. The above media were adjusted to pH 3.5 $\pm$ .1 with lactic acid.

PLATING

Prior to plating, the seven media to be used were melted and adjusted to pH 3.5 with normal lactic acid. A small amount of the medium was removed from the flask and the pH determined by the quinhydrone electrode. It was found that within a batch of media the pH could be kept constant but with each new batch of media it was necessary to check for pH, since a variation was found in the buffer strength, especially in the whey agars prepared in the laboratory. The samples of butter were plated in duplicate at a dilution determined by previous preliminary plating. The work was so organized that 3 samples of butter could be plated and the media poured within 15 minutes of time. Incubation was at 25°C. for 5 days.

RESULTS

In tabulating series one, 55 samples of butter were plated and in this group 14 were excluded because they contained less than 10 yeasts and molds per c.c.

In order to arrive at an average figure the yeast and mold count was converted into the logarithm of the number. In cases where the difference between duplicates exceeded 20 per cent in the number of colonies per plate, the butter was replated.

The results in table 1 indicate that wort, malt, and standard nutrient agar 5 per cent dextrose give similar results and that infusion agar plus 1 per cent lactose and whey agar prepared by rennet coagulation give the

TABLE 1

The logarithmic average of the yeast and mold count of 41 random samples of butter plated in duplicate

KIND OF MEDIA	NUMBER OF YEASTS AND MOLDS PER C.C.
Standard nutrient agar, 1% lactose	17,100
Standard nutrient agar	10,900
Dehydrated malt agar, Difco	20,600
Dehydrated wort agar, Difco	22,300
Standard nutrient agar, 5% dextrose	21,700
Infusion agar, 1% lactose	3,820
Whey agar—rennet coagulation	2,200

lowest counts. The butters used in series 1 were high in yeasts, the mold count being so low in many samples that only yeast colonies appeared on the plates.

In cases where the colonies on the plate appeared doubtful they were picked and examined microscopically; however, there was found no bacterial colonies on any of the doubtful plates.

TABLE 2

The logarithmic average of the yeast and mold count of 32 random samples of butter plated in duplicate

KIND OF MEDIA	NUMBER OF YEASTS AND MOLDS PER C.C.
Whey agar—rennet coagulation	712
Whey agar—acid coagulation	2,172
Whey agar dehydrated, Difco (batch 1)	4,157
Whey agar dehydrated, Difco (batch 2)	3,831
Standard nutrient agar, 5% dextrose	2,130
Wort agar dehydrated, Difco	3,684
Malt agar dehydrated, Difco	3,164

The results in table 2 confirm the results of table 1 the lowest count was obtained on whey agar prepared by rennet coagulation. It is of interest to note that the difference between two batches of dehydrated whey agar is slight and whey agar prepared by acid coagulation was superior to the whey agar prepared by rennet coagulation; the results obtained with standard nutrient agar plus 5 per cent dextrose was not as satisfactory as those in table 1.

In the second series the media were placed in position according to their count, that is the medium within a sample of butter that gave the highest yeast and mold count was placed first, the next highest, second and so on. The results are given in the following table:

Whey agar prepared by rennet coagulation out of a possible 34 times 27 times placed last, that is it showed the lowest yeast and mold count of media

TABLE 3

The distribution of placement of 34 samples of butter

MEDIA	FREQUENCY OF PLACING						
	Position						
	1	2	3	4	5	6	7
Whey—rennet	0	0	0	0	1	6	27
Whey—acid	4	0	2	4	9	14	1
Whey 1, Difco	8	9	5	8	2	2	0
Whey 2, Difco	8	12	6	4	3	1	0
SNA, 5% dextrose*	4	1	3	6	11	5	0
Malt, Difco	3	6	12	5	4	4	0
Wort, Difco	7	8	6	8	4	1	0

* 30 samples.

studied, or in other words whey agar prepared by rennet coagulation failed to yield as high counts as the other media.

Table 3 shows considerable overlapping of media and while it brings out that whey agar prepared by rennet coagulation was inferior, inasmuch as it produced the lowest yeast and mold count, it fails to show which media were superior, for no media consistently produced higher counts within the same sample of butter.

If, however, the frequency of position is totaled for the first three positions in respect to the other media in yielding the highest yeast and mold count of the 34 samples of butter studied then whey agar no. 2 dehydrated Difco yields the highest counts in that 26 or 77.5 per cent of the 34 butters are placed within the first 3 positions. Next in order of high counts are whey agar no. 1 dehydrated Difco 22 or 65 per cent, wort and malt agars each with 21 or 62 per cent, standard nutrient agar plus 5 per cent dextrose 8 or 23 per cent, whey agar prepared by acid coagulation 6 or 17 per cent and whey agar prepared by rennet coagulation none.

An analysis of these placings indicate, that in so far as the yeast and mold count of butter approximately the same relative values would be obtained with the following media: Whey agar dehydrated Difco, batches 1 and 2, and wort and malt dehydrated Difco. The media prepared in the laboratory yielded lower counts within the same samples that did the dehydrated media.

This confirms table 2 in which the media were compared on the basis of actual count.

In the two series of butters studied, four media were common to both series, whey agar rennet coagulated, standard nutrient agar plus 5 per cent dextrose, wort and malt agars dehydrated Difco. In tables 1 and 2 is shown that of the above four media wort agar dehydrated Difco yielded the highest average logarithmic yeast and mold count. The average logarith-

metric counts obtained on the media are represented as per cents taking the count obtained on wort agar dehydrated Difco as 100.

TABLE 4

Percentage comparison of butters in series 1 and 2 with dehydrated wort agar and the logarithmic average of yeast and mold count

MEDIUM	SERIES 1	SERIES 2
Whey—rennet coagulation	9.86	19.32
Standard nutrient agar, 5% dextrose	97.30	57.81
Malt agar dehydrated, Difco	92.37	85.88
Wort agar dehydrated, Difco	100.00	100.00

In both series the highest counts were obtained on dehydrated wort agar, the wort agar used in each series was of different batches prepared by the Digestive Ferments Company so that there was no significance as to its method of preparation or its composition. In the foregoing table in Series 1 little percentage difference is noted between standard nutrient agar plus 5 per cent dextrose, dehydrated malt agar, and wort agar; while in Series 2 a greater percentage difference is noted in the count obtained on each medium. This might be accounted for in the fact that lower yeast and mold count butters were used in Series 2 and that the mold count, because of lower dilutions became measurable, and also of variation in the preparation of the media in that in Series I and 2 not the same batch of whey agar rennet coagulated, or standard nutrient agar plus 5 per cent dextrose was used, though the same batch was used within a series.

TABLE 5

Cubic centimeters of normal lactic acid used to adjust 100 cc. of whey medium

MEDIA	NORMAL LACTIC ACID
Whey—rennet coagulation	11.0
Whey—acid coagulation	6.5
Whey dehydrated, Difco	2.0

Judging from the results procured on whey media there is noted a relationship between the degree of buffer of the medium and the yeast and mold count on the medium. Whey agar dehydrated showed higher yeast and mold growth than whey agar acid coagulation and whey agar rennet coagulation showed the least growth and had the highest buffer. Wort and malt agars which supported high counts had buffer values equal to whey dehydrated agar inasmuch as they required 2.0 cc. of normal lactic acid to adjust the pH to 3.5. The original hydrogen-ion concentration of the media was approximately the same varying from pH 6.4 to 6.8.

From the data were taken 10 random samples of butter and the actual yeast and mold counts on whey agar rennet coagulated, whey agar acid co-

TABLE 6

Typical yeast and mold counts on butter picked at random from the data

BUTTER NO.	WHEY RENNET	WHEY ACID	WHEY DIFCO	WORT DIFCO
S5	10	60	85	65
S1	45	310	310	230
C5	890	1,980	3,270	3,300
C4	10	40	470	60
B6	25	175	550	170
B8	185	1,465	2,245	2,530
5	150	55	700	510
S	1,910	6,860	16,240	15,800
C8	235	760	815	1,025
C11	3,350	5,300	7,650	6,100

agulated, whey agar batch 1 dehydrated Difco and wort agar dehydrated Difco, are recorded in table 6. The same trend is indicated in this table as in tables 1, 2, 3, and 4, showing the variation in yeast and mold count that is obtained within a sample of butter is dependent upon the medium used.

These studies are not completed inasmuch as there are other media to be compared and factors to be studied within the media already employed as to why the cause in variation in yeast and mold count.

CONCLUSIONS

From the data presented there can be concluded.

1. That whey agar prepared by rennet coagulation produced the lowest yeast and mold count.

2. That the difference between dehydrated agars prepared by Digestive Ferments Company (whey, malt, and wort) is slight.

3. That in order to procure comparative yeast and mold counts that either dehydrated whey, malt, and wort agars should be used.

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SEASONAL COMPOSITION OF PASTURE GRASSES

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During the past three years, 12 or more caged pasture areas have been clipped at monthly intervals. This paper reports the yield and analysis of grass from ten of these areas maintained during the growing seasons of 1930 and 1931. Four areas were in bench land pasture that had been seeded to oats in November, 1929. The other areas were on alluvial land that had been in pasture less than three years. The soil is nearly neutral in reaction, high in phosphorus and apparently in available nitrates, due to manuring. The prevailing vegetation in early spring was White Dutch clover (*Trifolium repens*) except in the oat plots. Here much hop clover (principally *Trifolium procumbens*) occurred with the oats the first year. These two crops gave way in May to Dallis (*Paspalum dilatatum*) and Bermuda (*Cynodon dactylon*) grasses with the white clover reappearing in September and October. Considerable rye grass (*Lolium multiflorum*) was found in spring and carpet grass (*Axonopus compressus*) in the summer, especially on the bench land.

Eight of the cages were 3×7 feet and two 2×2 feet in size. The entire area was closely cut with a grass hook, the yield recorded and individual samples taken for analysis. Analyses were by standard methods adopted by the Association of Official Agricultural Chemists. Another portion of each sample was dried to constant weight at room temperature and from this the hay yield determined. The nutritive value was calculated from the coefficient of digestibility of peas and oats for the cuttings previous to June and of Bermuda grass for the latter cuttings.

A remarkable similarity of composition of the samples cut the same date was noticed both seasons, with alluvial land yielding more grass slightly higher in protein and moisture content. Comparing 1930 and 1931 the monthly composition was again very similar, although 1931 average yield of air-dry hay was 28 per cent greater for the season. Therefore all samples cut the same month have been averaged together as shown in table 1. Four samples of clover and rye grass cut January 15, 1931, are included to show the high moisture and protein content at that season, likewise nine samples for February, 1931. The difference between 20 and the number of samples listed for other months is due to destruction by cattle. The yearly average includes all normal grass samples of the season. Because of dry weather

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TABLE 1

Composition of pasture grasses cut monthly 1930–1931

DATE OF GROWTH	NO. SAMPLES	RAIN-FALL	HEIGHT	GRASS PER ACRE	HAY PER ACRE	DRY MATTER IN 100 LBS. GRASS	PER CENT OF DRY MATTER						T.D.N. PER 100 LBS. GRASS (EST.)
							Crude protein	P.E.	Fat	N.F.E.	Fiber	Ash	
		inches	*inches*	*pounds*	*pounds*	*pounds*							*pounds*
Jan. '31	4		5	–	–	14.55	26.92	± 2.38	4.92	38.39	16.52	13.24	9.14
Feb. '31	9	4.46	4.6	7560	1316	16.08	22.95	± 2.01	3.93	38.10	23.71	10.44	10.08
Mar.	15	4.55	4.9	8350	1442	15.87	22.54	± 3.30	3.83	46.25	28.92	9.11	10.20
Apr.	19	2.73	9.2	11,298	2261	18.57	18.86	± 3.55	4.01	42.81	23.74	10.08	11.84
May	18	4.60	4.3	4738	1706	22.68	18.68	± 2.03	3.25	38.21	26.80	12.65	13.46
June } July }	19	3.26	5.0	3726	1407	33.74	13.74	± 1.66	2.70	46.66	26.84	10.07	16.96
Aug.	20	4.95	6.0	7271	2184	24.18	12.86	± 1.67	3.62	44.64	28.65	10.72	11.75
Sept.	20	5.02	5.4	6024	1844	27.29	10.96	± 1.16	3.36	46.15	29.54	10.00	13.11
Oct.	20	5.98	5.0	3588	1213	28.64	11.45	± 1.05	3.53	44.52	29.32	11.05	13.55
1930	68	34.52		39,566	9,937	21.70	15.21		3.03	42.94	27.12	11.70	13.20
1931	95	32.10	39.5	49,979	12,763	24.67	16.81		3.45	44.97	25.31	9.46	13.38

and slow growth both seasons, the June cutting was delayed and combined with July. The date of growth is for the majority of the month listed.

The seasonal trend is definitely from high moisture and crude protein to low moisture and crude protein at mid-season with a corresponding increase in crude fiber and nitrogen-free extract. The result is a gradual increase in total digestible nutrients per 100 pounds of grass. Fat and total ash apparently vary little as the season advances although individual fluctuations occurred. Rate of growth is more rapid in the early spring, probably due to the storage of food in plant roots and secondarily to favorable temperature conditions. Total rainfall for each period of growth apparently has less effect on total yield than one would expect. The greatest yield occurred in the period of least rainfall and smallest yield with the greatest rainfall.

Distribution of rainfall and minimum temperatures are probably of great importance as affecting growth and indirectly composition. Rapidly growing plants as oats in early spring were quite similar to clover samples in analyses as previously noted by the author (1).

Variation in protein content of various samples was least in late summer, however.

Dry matter of grass from top dressed areas with 100 pounds Chilean nitrate of soda in 1930 averaged 14.34 per cent crude protein; similar areas with nitrate plus 150 pounds 18 per cent superphosphate gave 13.59 per cent crude protein, while untreated areas gave 13.78 per cent crude protein. With a doubled application in 1931 for the same areas, the respective crude protein figures were 16.61, 16.02, and 16.17 per cent. of the dry matter. Increasing the available supply of plant food on good land thus had no effect on protein content of the grass. This does not apply to poor land, however.

Eighteen composite samples of dried grass hay representing cuttings February to May inclusive averaged 0.69 per cent phosphorus and 1.21 per cent calcium. This is similar in phosphorus but slightly lower in calcium than the average legume hay. It certainly would not indicate a mineral deficiency for this pasture grass as given by Orr (2).

From a feeding standpoint, one may conclude that light feeding of home grown grains in early spring and heavier feeding of higher protein feeds in late summer should supplement similar pasture grass. However, 40 to 50 per cent of the nitrogen in young grass is in the form of non-proteins. The greater part of these non-proteins consists of amino-acids. The result is that the percentage of protein as given in the table is probably much too high for spring months. That criticism applies to English analyses and all others of frequently clipped pastures. As the season or maturity progresses practically all the amino acids are used to build up proteins; consequently it appears that the percentage of protein has decreased

markedly. The decrease is not as large as the figures indicate and this should be considered when comparing such data. In that respect these data coincide closely with that of Hopper and Nesbitt (3) for crude protein content of unclipped and clipped grasses and are in agreement with other material reviewed by them with one exception. Schutt, Hamilton, and Selwyer (4) found protein content to increase with season. Ellenberger, Newlander and Jones (5) found crude protein to vary little in grass cut monthly although the ordinary hill pasture gave a lower protein content than rich pasture. The results of Salter and Yoder (6) showing a decided increase of crude protein for 1929 as season advanced and with fertilization is in direct disagreement with our results.

These disparities can possibly be explained by the difference in type of vegetation and rate of growth here in the south from that of the east and north. We can at least agree with these workers that proportion of nutrients in pasture grass generally approaches a medium protein ration or high quality legume roughage.

Conclusions

1. There is little difference in chemical composition of grass from fertile pastures cut the same date.

2. The season of year or rate of growth is more important than type of vegetation in influencing the protein and fiber content of monthly clipped pasture grass.

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THE VITAMIN A ACTIVITY OF BUTTER PRODUCED BY GUERNSEY AND AYRSHIRE COWS*

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The natural color of butter is known to vary with breeds of cows. This color is due almost entirely to carotin. Since carotin is now recognized as a precursor of vitamin A and as such acts biologically as vitamin A, it might seem logical to assume that butter rich in this pigment, might also possess greater vitamin A activity than the paler colored butters. If this were true, butter produced by such breeds as Jersey and Guernsey cows should possess greater vitamin A potency than that produced by Holstein and Ayrshire cows. However, butter does not owe its entire vitamin A activity to carotin. Since Stephenson (1) reported decolorizing butter with charcoal without apparently impairing its vitamin A activity, it must be assumed that vitamin A (*per se*) is present in butter. This fact was later confirmed by Morton and Heilbron (2) who demonstrated by spectroscopic analyses that both carotin and vitamin A are present in butter. Similar results were reported by Moore (3) who also demonstrated that cows were able to convert carotin into vitamin A. With carotin and vitamin A contributing to the vitamin A activity of butter, the variation in color of butters produced from different breeds may be less significant than generally supposed.

There has been little work reported on the relationship between vitamin A activity for butter and breeds of cows. Holmes (4) studied the vitamin A potency of butterfat from Holstein, Jersey, Ayrshire, Devon and Dutch Belted cows, but considered his data insufficient to permit any definite conclusions. More recently, Davis and Hathaway (5) claim that under the conditions of their experiments, the difference in vitamin A content of milk from Holstein, Ayrshire, Jersey or Guernsey breeds is insignificant. Moore (3) states that when carotin is available in adequate amounts in the diet, the degree of pigmentation of butterfat is determined by breed. Butterfat derived from Jersey cows gave a yellow color value twice that obtained with butter produced by Shorthorn cows under similar nutritive conditions. Because of the fact that Ayrshire milk is relatively light in color while Guernsey milk is decidedly yellow in color, these two breeds were selected for a study to determine the relative vitimin A activity of butters produced under similar nutritive conditions.

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EXPERIMENTAL PROCEDURE

Two Ayrshire and four Guernsey cows were used in this experiment. All six cows had been fed the same since the beginning of their lactation period, having been on blue grass pasture throughout the spring and summer. In addition to the pasture, they received a balanced grain ration, and, during the short pasture period of July and part of August were fed small amounts of alfalfa hay and silage. From August fifteenth to October first, these cows were restricted to blue grass pasture, corn silage and a grain ration consisting of 400 pounds ground white corn, 200 pounds ground oats and 100 pounds linseed oilmeal. On October first, the six cows were divided into 3 groups as follows: Lot 1, two Guernseys; Lot 2, two Ayrshires and Lot 3, two Guernseys. By this division, butter samples were secured from two Guernsey groups and one Ayrshire group, thus making it possible to not only compare the vitamin A activity of butters from the two breeds but also to check possible variations within a breed. On October first and second, composite samples of milk (equal amounts from each cow) were collected from each group, the cream separated and churned into butter. These butter samples were kept in cold storage and portions removed as needed.

These three samples of butter were then subjected to biological assays to determine the vitamin A activity, using the curative method. Young albino rats were used as experimental animals. The number of animals employed for each dosage ranged from four to eight, the sex distribution being equal. Each rat was kept in an individual cage of a type which prevented access to excreta. The vitamin A deficient ration was given ad libitum in modified McCollum type feeding cups. The vitamin A deficient ration was composed of: Casein (extracted) 18 per cent; ground white corn, 25 per cent; dextrin, 36 per cent; lard, 10 per cent; salt mixture (McCollum's 185), 4 per cent; and agar, 2 per cent and yeast (Fleischman's Irradiated) 5 per cent. During the preliminary depletion period, the rats were restricted to the vitamin A deficient ration until the storage of vitamin A in the animals was exhausted, as evidenced by definite symptoms of vitamin A deficiency. At this point, usually 2 to 3 weeks, the animals were given test doses of butter in amounts of 30 ,50, 75, 100, 150 and 200 mgs. daily. These doses were mixed with small amounts of the basal ration in small glass dishes and fed to the individual rats. The test period was eight weeks. Weekly records of weight of the animals and food consumption were kept.

The results of these tests are given in Chart 1. It is to be noted that the biological responses to these three samples of butter are practically the same. This would indicate that the comparative vitamin A activity of butters from the two breeds is no different from that within the same breed. This was rather surprising in view of the disparity between the color of the

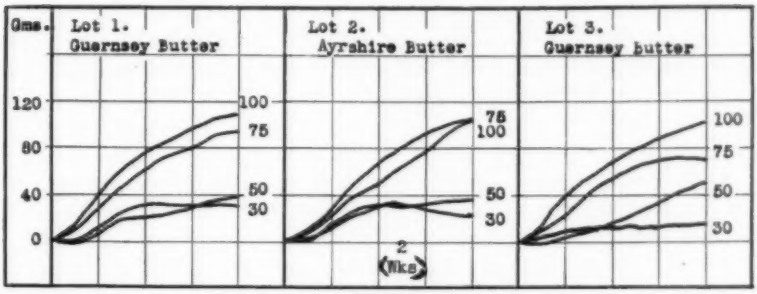

CHART 1

Composite growth curves of groups of rats showing the response to butters from one group of Ayrshire cows and two groups of Guernsey cows. The numbers opposite each curve represent the daily dosage of butter in milligrams. The vitamin A activity of the three samples of butter was apparently the same.

butters. The carotin content of the butter samples from Lots 1, 2, and 3 was 4.0, 1.8 and 3.6 mgs. respectively per hundred grams of butterfat as determined by Palmer's method (6).

DISCUSSION

The results of these experiments indicate that there is no appreciable difference between the vitamin A activity of butter produced by Guernsey and Ayrshire cows when fed under identical nutritive conditions. This might at first appear to be in agreement with Davis and Hathaway (5) who concluded that there was no difference in the vitamin A content of milk produced by different breeds of cows under similar feeding conditions. However, it should be pointed out that Davis and Hathaway were working with whole milk of variable fat content, while in these experiments, the tests were made on butter.

Since the vitamin A potency of milk resides in its butterfat, it is possible to translate the results of their tests with milk into a comparable vitamin A value for the butterfat. The variations in percentage of fat in the milk used by these investigators were reported as follows: Holstein 3.1 to 3.55; Ayrshire 4.05 to 4.3; Guernsey, 4.7 to 4.8; and, Jersey, 5.2 to 6.5. Since each cubic centimeter of milk gave comparable growth response in rats, it is apparent that 31 to 33 mgs. of Holstein butterfat were equal to 40–43 mgs. of Ayrshire butterfat; or to 47–48 mgs. of Guernsey butterfat; or to 52–65 mgs. Jersey butterfat. In other words, these data would indicate that butter churned from these samples of milk would vary considerably in vitamin A activity, Holstein being the highest and Jersey the lowest. From this it might be concluded that as the natural color increases with breed, the vitamin A value decreases.

However, on the basis of our results which showed uniform vitamin A content for Guernsey and Ayrshire butterfat it is apparent that under similar feeding conditions, the higher the percentage of butterfat in milk the greater will be its vitamin activity.

SUMMARY

1. Comparisons were made of the vitamin A activity of butters from one group of Ayrshire cows and two groups of Guernsey cows, fed under identical nutritive conditions.

2. The wide variation in color of these butter samples was shown by their carotin content. The Guernsey butters contained 3.6 and 4.0 mgs. of carotin per 100 grams of butterfat while the Ayrshire butter contained only 1.8 mgs. of carotin.

3. The vitamin A activity of these samples of butter was found to be similar regardless of breed of cows or color of butter.

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THE SOURCE OF THE VOLATILE ACIDITY PRODUCED IN MILK BY THE CITRIC ACID FERMENTING STREPTOCOCCI

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The early studies at the Iowa Agricultural Experiment Station on the organisms normally present in good butter cultures showed (a) that the one type (*Streptococcus citrovorus* and *Streptococcus paracitrovorus*) produced comparatively high volatile acidities in milk, (b) that the volatile acidities produced were greatly increased by adding citric acid to the milk, and (c) that the volatile acidities of the cultures which developed very little acid in milk (*S. citrovorus*) were somewhat increased by adding lactic acid to the milk while the volatile acidities of cultures which developed some lactic acid (*S. paracitrovorus*) were not significantly increased by the addition of lactic acid.[1] These results were interpreted as indicating that citric acid is the important source of the volatile acidity produced in milk by this type of organism but that volatile acidity can also be produced from lactic acid, either that added to the milk (in the case of *S. citrovorus*) or that produced in the milk (in the case of *S. paracitrovorus*). If this interpretation is applied to butter cultures it suggests that the citric acid fermenting streptococci produce volatile acidity from the citric acid normally present in the milk and also, to a certain extent, from the lactic acid produced by *Streptococcus lactis*.

An attempt to determine whether or not the volatile acids that appeared to be formed from lactic acid by the citric acid fermenting streptococci normally present in butter cultures were the same as those formed from citric acid indicated that lactic acid is probably not a source of volatile acids with these organisms although it definitely increases the volatile acidity production in milk. Results of significance in connection with this point are presented herein.

GENERAL PROCEDURE

Various cultures of the citric acid fermenting streptococci (*S. citrovorus* and *S. paracitrovorus*) were grown (a) in sterilized, freshly drawn milk and (b) in sterilized, freshly drawn milk to which various acids had been added. The acids first used were citric and lactic; later tartaric acid and finally phosphoric and sulphuric were included.

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Freshly drawn milk was selected as the basic medium after extended preliminary trials with beef infusion bouillon modified by the addition of phosphate and yeast had shown that considerable volatile acid (propionic or propionic and acetic) was present in the distillate from the sterile uninoculated medium; milk fermented spontaneously or with a butter culture (to remove the citric acid present) and then distilled (to drive off the volatile acid), neutralized and sterilized was also found to be less satisfactory than freshly drawn milk.

In adding the various acids to the milk the following preparations were used: citric acid, c.p. crystals; lactic acid, approximately 85 per cent; tartaric acid, c.p. crystals; phosphoric acid, approximately 85 per cent; sulphuric acid, approximately 95 per cent. The desired amount of acid was weighed out and dissolved in a small quantity of water, the solution sterilized and then added to the inoculated milk; usually the organisms were permitted to grow several hours in the milk before adding the acid.

The volatile acidities of the uninoculated milk, with or without added acid, were regularly low.

A culture was examined for volatile acid production by distilling 1000 ml. with steam, after the addition of 50 ml. n/1 H_2SO_4, until 1000 ml. of distillate were secured and then titrating 50 ml. of the distillate with n/10 NaOH, using phenolphthalein as an indicator; the milliliters of n/10 NaOH which would have been required for the 1000 ml. of distillate were then calculated. The remaining 950 ml. of distillate were used for the preparation of a barium salt; when the acid present in the 950 ml. of distillate was insufficient to give a satisfactory amount of barium salt, the distillation was continued and the additional distillate included in the preparation. After the purification and drying of the salt, the percentage of barium contained was determined and the solution of volatile acids secured, when the barium was precipitated, was examined by a modified Duclaux procedure.[2] The barium values secured on salts prepared from commercial acetic or propionic acid agreed closely with the theoretical values.

RESULTS OBTAINED

Representative data on the volatile acidities produced by the citric acid fermenting streptococci in freshly drawn milk and in freshly drawn milk to which various acids had been added are given in table 1; the barium values of the volatile acids are also included. The results show that all the acids used increased the volatile acid production in milk cultures of most of the organisms studied. Citric acid gave distinctly greater increases than any of the other acids and, in certain concentrations, surprisingly high volatile acidities were secured with some of the organisms. All of

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TABLE 1

Volatile acidities produced by the citric acid fermenting streptococci in milk alone and in milk with various acids added

LOT OF MILK USED	ACID ADDED		TOTAL ACIDITY ML. N/10 NaOH FOR 9 GM. MILK	VOLATILE ACIDITY ML. N/10 NaOH FOR ONE LITER OF DISTILLATE**	% BARIUM IN BARIUM SALT OF VOLATILE ACID		
	Kind	Approx. %*			A	B	Ave.
			Culture 1				
1	None		2.5	39			
1	None		2.5	40	52.74	52.79	52.765
5	None		3.2	42			
5	None		3.2	42	53.16	53.13	53.145
1	Lactic	0.3	5.4	64			
1	Lactic	0.3	5.3	59	52.82	52.78	52.80
4	Lactic	0.6	7.1	50			
4	Lactic	0.6	6.8	52	53.01	52.99	53.00
5	H_2SO_4	0.2	5.8	57			
5	H_2SO_4	0.4	8.5	58	53.48	53.31	53.395
5	H_3PO_4	0.3	7.2	54			
5	H_3PO_4	0.6	11.6	60	53.30	53.04	53.12
5	Tartaric	0.3	4.9	54			
5	Tartaric	0.6	6.4	60	53.58	53.56	53.57
1	Citric	0.3	4.2	86			
1	Citric	0.3	4.2	82	53.23	53.18	53.205
			Culture 2				
2	None		3.2	50			
2	None		3.1	50	53.26	53.29	53.275
2	Lactic	0.3	6.1	65	53.29	53.23	53.26
2	Citric	0.3	7.3	144	53.65	53.65	53.65
			Culture 3				
4	None		4.2	46			
4	None		4.3	47	53.30	53.32	53.31
4	Lactic	0.6	8.2	66			
4	Lactc	0.6	8.2	63	53.56	53.62	53.59
4	Citric	0.3	8.1	127	53.72	53.71	53.715
4	Citric	0.6	9.1	172	53.85	53.79	53.82
			Culture 4				
1	None		6.3	78			
1	None		6.2	82	53.18	53.19	53.185
1	Lactic	0.3	8.2	75			
1	Lactic	0.3	8.2	74	53.03	53.08	53.055
5	H_2SO_4	0.2	8.9	80			
5	H_2SO_4	0.4	9.5	68	53.76	53.80	53.78
5	H_3PO_4	0.3	9.5	66			
5	H_3PO_4	0.6	12.5	70	53.45	53.52	53.485
5	Tartaric	0.3	7.5	76			
5	Tartaric	0.3	7.9	71	53.67	53.67	53.67
1	Citric	0.3	9.1	145			
1	Citric	0.3	8.2	173	53.46	53.41	53.435

TABLE 1.—*Continued*

LOT OF MILK USED	ACID ADDED		TOTAL ACIDITY ML. N/10 NaOH FOR 9 GM. MILK	VOLATILE ACIDITY ML. N/10 NaOH FOR ONE LITER OF DISTILLATE**	% BARIUM IN BARIUM SALT OF VOLATILE ACID		
	Kind	Approx. %*			A	B	Ave.
			Culture 5				
1	None		2.0	18	51.44	51.46	51.45
1	None		2.1	11			
4	None		2.6	12			
4	None		2.5	13			
5	None		2.3	15			
5	None		2.5	18			
1	Lactic	0.3	5.4	60	53.28	53.35	53.315
1	Lactic	0.3	5.1	60			
4	Lactic	0.6	6.8	55	53.55	53.44	53.495
4	Lactic	0.6	6.8	60			
5	H_2SO_4	0.2	6.0	57	53.65	53.59	53.62
5	H_2SO_4	0.4	8.4	55			
5	H_3PO_4	0.3	7.5	51	53.32	53.45	53.385
5	H_3PO_4	0.6	12.1	58			
1	Citric	0.3	4.7	72	53.32	52.90	53.11
1	Citric	0.3	4.7	68			
			Culture 6				
2	None		2.3	18	52.01	52.03	52.02
2	None		2.3	14			
4	None		2.2	10			
5	None		2.2	16			
5	None		2.2	13			
2	Lactic	0.3	5.2	58	53.32	53.39	53.355
4	Lactic	0.6	6.5	44	52.73	53.06	52.895
4	Lactic	0.6	6.2	46			
5	H_2SO_4	0.2	5.6	42	53.59	53.68	53.635
5	H_2SO_4	0.4	7.6	57			
5	H_3PO_4	0.3	7.1	44	53.66	53.66	53.66
5	H_3PO_4	0.6	11.8	54			
2	Citric	0.3	5.4	74	53.12	53.17	53.145
			Culture 7				
3	None		2.7	20	52.08	52.08	52.08
3	None		2.7	18			
3	Lactic	0.3	5.3	56	53.19	53.23	53.21
3	Citric	0.3	5.9	70	53.26	53.21	53.235
			Culture 8				
3	None		2.7	26	52.49	52.86	52.675
3	None		2.6	22			
3	Lactic	0.3	5.6	56	53.42	53.44	53.43
3	Lactic	0.6	6.7	57	53.38	53.39	53.385
3	Citric	0.3	5.4	68	53.00	53.04	53.02

TABLE 1.—*Continued*

LOT OF MILK USED	ACID ADDED		TOTAL ACIDITY ML. N/10 NaOH FOR 9 GM. MILK	VOLATILE ACIDITY ML. N/10 NaOH FOR ONE LITER OF DISTILLATE**	% BARIUM IN BARIUM SALT OF VOLATILE ACID		
	Kind	Approx. %*			A	B	Ave.
			Culture 9				
4	None		4.7	53	53.56	53.54	53.55
4	None		4.7	48			
4	Lactic	0.3	7.4	66	53.22	53.15	53.185
4	Lactic	0.6	8.5	66	53.39	53.25	53.32
4	Lactic	0.6	8.4	60			
4	Citric	0.3	8.2	126	53.62	53.57	53.595
			Culture 10				
3	None		5.2	51	53.01	52.99	53.00
3	None		5.3	51			
3	Lactic	0.3	7.8	61	53.28	53.31	53.295
3	Citric	0.3	9.3	151	53.58	53.64	53.61
			Culture 11				
3	None		4.5	44	53.05	53.11	53.08
3	None		4.4	44			
3	Lactic	0.3	7.3	58	53.15	53.01	53.08
5	Lactic	0.6	9.3	58	53.11	53.01	53.06
3	Citric	0.3	7.9	142	53.67	53.62	53.645
3	Citric	0.6	9.3	188	53.63	53.61	53.62

* Concentrations of acids are given under General Procedure.

** Calc. from titration values for 50 ml. quantities of the distillates.

the acids other than citric gave about the same general increase in volatile acid production.

The influence of the addition of acid in increasing the volatile acidity produced in milk varied greatly with the different cultures and was more or less correlated with the titrable acidity produced by the cultures. With cultures producing comparatively small amounts of titrable acidity in milk (for example, cultures 5 and 6) there was comparatively little volatile acidity produced in milk alone and, when acid was added to the milk in which these cultures were grown, a great increase in the volatile acid production occurred. With the cultures producing comparatively large amounts of titrable acidity in milk (for example, cultures 3, 4, 9, 10 and 11) there was considerable volatile acidity produced in milk alone and, when acid other than citric was added to the milk in which these cultures were grown, comparatively little or no increase in the volatile acid production occurred. Culture 4 gave a titrable acidity and also a volatile acidity distinctly higher than those produced by the other cultures and with it none of the added acids except citric significantly influenced the volatile acid

production in milk. In general, the cultures that gave the very high volatile acidities in milk to which 0.3 per cent citric acid had been added were those that produced comparatively high titrable acidities in unmodified milk.

The percentages of barium in the barium salts prepared from the volatile acid produced in milk, with or without added acid, varied from 51.45 to 53.82 per cent. The barium values secured on the volatile acid distilled from milk that had considerable titrable acidity at the time of distillation, either through the addition of acid or as a result of the growth of the organism added, showed only small variations and differed only slightly from the theoretical for acetic acid (53.78). There was a general tendency for the barium values to be slightly higher with the high volatile acidities obtained from milk plus citric acid than with the lower volatile acidities obtained from milk plus other acids. Considerable variation occurred in the barium values secured on the volatile acid produced in unmodified milk by the different cultures and, commonly, the lower the volatile acid production the lower the barium values. With the two cultures producing the lowest volatile acidities in milk (cultures 5 and 6) the barium values were enough lower than the theoretical for barium acetate to suggest that considerable acid of a molecular weight higher than acetic was included. In general, the values secured by a modified Duclaux method on the volatile acids that were freed when the barium was precipitated, agreed with the barium values.

The preliminary trials with the modified beef infusion bouillon and with milk that was fermented, distilled and neutralized (referred to in the General Procedure) are of interest in connection with the interpretation of the results secured on freshly drawn milk. With these media the addition of acid gave significant increases in volatile acidity only in the case of citric; in the bouillon glycollic, lactic, beta-hydroxypropionic, succinic, malic, and tartaric acids did not give an appreciable increase and in the fermented milk lactic acid failed to give an appreciable increase. When citric acid was added to the bouillon the barium values on the volatile acid indicated largely acetic acid while when acids other than citric were added the barium values indicated largely or entirely propionic acid.

DISCUSSION OF RESULTS

The results secured confirm the previously reported observation that the addition of lactic acid to milk cultures of the citric acid fermenting streptococci increases the volatile acidity produced and also show that tartaric, phosphoric and sulphuric acid give essentially the same increases in volatile acidity. Since presumably volatile acids cannot be formed from such acids as phosphoric and sulphuric, it is probable that lactic acid also is not a source of volatile acid and that it influences the amount of volatile

acid produced only in the same manner as other acids. This influence apparently involves the establishment of a hydrogen ion concentration that is very favorable for the production of volatile acid in milk by these organisms and may include the freeing of citric acid from its salts.

The relationship of titrable acidity to the production of volatile acidity in milk is also emphasized by the greater volatile acidities produced in unmodified milk by the cultures of citric acid fermenting streptococci that increased the titrable acidity considerably than by those that increased it but little.

The importance of citric acid as a source of volatile acidity is shown by the great increases secured through its addition to freshly drawn milk inoculated with the citric acid fermenting streptococci and also by the increases secured through its addition to such media as modified beef infusion bouillon and fermented milk which failed to give increases in volatile acidity when lactic acid or various other acids were added.

The suggested action of lactic acid in increasing the volatile acidity produced in milk by the citric acid fermenting streptococci explains: (a) the comparatively small increases in volatile acidity secured when lactic acid is added to milk cultures of these organisms; (b) the limited quantity of volatile acid produced in butter cultures in the presence of comparatively large amounts of lactic acid, a point that is difficult to understand if lactic acid is a source of volatile acid; and (c) the failure to secure an increase in the volatile acid production when lactates are added to milk cultures of the citric acid fermenting streptococci.[3]

SUMMARY

Lactic acid increased the volatile acidity produced in milk cultures of the citric acid fermenting streptococci, but similar increases were secured with tartaric, phosphoric and sulphuric acid and, accordingly, it appears that lactic acid is not a source of the volatile acidity produced by these organisms. The influence of the various acids presumably involves the establishment of a hydrogen ion concentration that is very favorable for the production of volatile acid in milk by these organisms and may include the freeing of citric acid from its salts. The effect of acids in increasing the volatile acidity produced in milk varied greatly with different cultures and was greatest with cultures that produced comparatively small amounts of titrable acidity, and least with cultures that produced comparatively large amounts of titrable acidity. Added citric acid gave such large increases in the volatile acid production of the organisms in milk cultures that it is evidently a source of volatile acid.

[3] See ref. (1).

The importance of citric acid as a source of volatile acid with the citric acid fermenting streptococci was also shown by striking increases in the volatile acid production when citric acid was added to a modified beef infusion bouillon or to milk which had been fermented to remove the citric acid and then distilled to drive off the volatile acids; with the bouillon glycollic, lactic, beta-hydroxypropionic, succinic, malic and tartaric acids failed to give an increase in volatile acid while with the fermented milk lactic acid failed to give an increase.

THE SIGNIFICANCE OF COLON BACTERIA IN MILK, WITH SPECIAL REFERENCE TO STANDARDS[1]

JAMES M. SHERMAN AND HELEN UPTON WING
Cornell University, Ithaca, New York

INTRODUCTION

The subject of colon bacteria in milk has for years received so much attention that the topic is indeed somewhat threadbare. Nevertheless, there continues to be sufficient diversity of opinion, to make this shopworn problem of present vital concern to the dairy industry. The "colon test" has been used for a variety of purposes in dairy work, and therefore the purpose of this discussion should be made clear at the outset. We are here interested in the application of the test as a sanitary index to raw milk as it is received from the producer or as it is delivered to the consumer by the raw milk distributor. Other uses of the colon test, such as controlling the efficiency of pasteurization, checking the recontamination of milk in the plant after pasteurization, and as an index of the fitness of milk for the manufacture of certain milk products, represent quite different subjects which will not be dealt with in this paper.

When we speak of the "colon organisms" of milk, it is generally understood to mean that class of bacteria known as the "coli-aerogenes" group or the Escherichia-Aerobacter group, so named because of its two leading species, *Bacterium coli* (*Escherichia coli*) and *Bacterium aerogenes* (*Aerobacter aerogenes*). These bacteria are not pathogenic (except in a few very exceptional cases), but they are related to such pathogenic species as the typhoid and dysentery organisms. While the colon bacteria in milk are not in themselves dangerous from the standpoint of health, their occurrence in significant numbers is considered by some as of sanitary importance, because of the belief that their presence in milk indicates pollution with fecal matter.

This brings us to the subject of the sources of the colon bacteria—where they exist and grow in nature. It has just been said that they are sometimes looked upon as being of fecal origin. This is true. Members of this group of bacteria are natural inhabitants of the intestinal tract of warm-blooded animals, and they are found in large numbers in manure. We are not here concerned with technical distinctions between the different members of this group of organisms, but it may be stated that many of the varieties which belong to this group are not of fecal origin but are asso-

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[1] Paper presented at the twenty-fifth annual convention of the International Association of Milk Dealers, Detroit, Michigan, October 18, 1932.

ciated in nature with vegetation and the soil. They are found on plants and grains, and most of the mixed feeds which the dairymen use contain bacteria of this type in large numbers. Because of these facts it is generally recognized that the presence of the "colon group" of organisms in milk does not necessarily imply fecal pollution.

When viewed from the sanitary standpoint another important fact must be remembered. Milk is a very good medium for the growth of these bacteria, so that they may sometimes increase and subsequently occur in large numbers in milk which was not initially contaminated with undue amounts of undesirable substances such as manure, feeds, bedding, and soil.

Concerning the undesirability of the colon bacteria in milk there can be but one view. They are thoroughly undesirable. They find their way into milk with substances which should be as rigidly excluded as is possible. If allowed to grow extensively in milk, they give rise to taints and abnormal flavors. In the fermentation of the milk sugar they produce gas and other objectionable by-products, which makes them one of the worst enemies of the manufacturer of butter, cheese, and other milk products.

Thus far nothing has been said to which exception could be taken, regardless of one's views concerning the desirability of applying a standard which would limit the number of bacteria of the colon type which may occur in raw milk. On this point there is a considerable difference of opinion. In New York, neither the State Department of Health nor the New York City Department of Health has seen fit to apply this test as a regular means of judging the sanitary fitness of raw milk. Also, the American Public Health Association does not recognize the colon test as an official method in its Standard Methods of Milk Analysis. The view of these bodies appears to be that the sanitary significance of colon bacteria in milk is not clear; and that the information revealed from such a test is little, if any, more than that obtained by the total bacterial count. On the other hand, there are many who believe that the colon test has a distinct sanitary value. There are many communities where this test is used locally, while it has been applied on a state-wide basis in a few instances. Again, we frequently find the colon test coming into prominence whenever there is a special investigation of a milk supply.

METHODS

Before taking up the experimental data, a few words should be said about methods of determining the numbers of organisms of the colon group in milk. At the beginning of this work we tried Endo agar, eosin-methylene blue agar, a synthetic Endo agar, and some synthetic media of our own. Quantitative platings were made with these agars of samples of milk, and with twelve stock cultures of different strains of the Escherichia-Aerobacter organisms. All of the media proved satisfactory,

yielding results which came well within the expected experimental error. It was decided to use Endo agar, since that was the most commonly used medium for this purpose at the time these experiments were started.

While the presence and numbers of organisms of the colon group may be determined with equal accuracy with a variety of media, there are nevertheless some pitfalls. For example, the standard lactose broth fermentation tube, as used in water work, is not reliable when applied to milk. We have observed numerous instances in which milks which gave comparatively high colon counts on Endo agar failed to give positive tests when inoculated into lactose broth. The reason for this is probably that in this medium the lactic acid bacteria of milk sometimes grow with sufficient rapidity to inhibit the colon types before they reach numbers sufficient to produce a detectable amount of gas.

Another word of warning concerning methods is not out of place. Newly suggested media should not be used unless their value has been thoroughly established. An outstanding instance may be cited: There has recently been recommended for the detection of colon organisms a lactose broth containing crystal violet. The purpose of the crystal violet is to inhibit other bacteria without affecting the colon types. Dr. Stark and Mr. England, of our department, have found that while stock culture of *Bacterium coli* appear to do well in this medium a large proportion of strains freshly isolated from feces are markedly inhibited by it.

We have had the opportunity of reading a paper on colon bacteria in milk, just recently prepared for publication, in which the workers used standard lactose broth and crystal violet broth, two methods, neither of which is reliable for this purpose.

PRACTICAL EXPERIENCE WITH THE COLON TEST

A survey was made which included 450 samples of raw milk from producers located in central New York. All of this milk was being utilized in cities within New York State as fluid milk. Most of the samples were taken as the milk was delivered at plants or collecting stations, though some were obtained at the farms. All of the milk samples were examined for total bacteria by the plate culture method, and also tested for the number of bacteria of the colon group. Of the 450 samples, 102 contained less than 10,000 bacteria by the standard plate culture method; 207 contained between 10,000 and 100,000; 63 between 100,000 and 300,000; while 78 contained more than 300,000 per cc.

It is fair to ask just what hardships would be imposed upon a probably representative group of better than average dairymen if a colon standard for milk were adopted and rigidly enforced.

A standard which has been established in a few municipalities requires that the raw milk, as delivered to the milk plants, shall contain less than

100 colon bacteria per cc. If this criterion were applied to the milks studied by us, it is found that 11 per cent of the samples containing less than 10,000 bacteria per cc. would be condemned. Of those having total counts between 10,000 and 100,000 the percentage would be 50; between 100,000 and 300,000, 73 per cent; while 63 per cent of the milks containing more than 300,000 total count would be condemned on the basis of the colon count. (See table 1.) Practically every one will agree that we are not justified at the present time in considering a standard which would exclude such a large proportion of our market milk, which we have fairly good reason to consider satisfactory from the sanitary point of view.

A much more reasonable standard, and one which has worked with apparent satisfaction in some localities, is to limit the colon count to less than 1,000 per cc. of the raw milk. If this standard is applied to the milks under discussion, we find that none of those with less than 10,000 bacteria per cc. would be condemned; 10 per cent between 10,000 and 100,000; 33 per cent between 100,000 and 300,000; and 45 per cent of those containing over 300,000 bacteria per cc. of milk. These results are also presented in table 1. When these figures are reviewed, it is clear that such a standard would not work great hardship on dairymen who are now producing milk with a total bacterial count of less than 100,000 per cc. Among the other producers, however, more than one-third of the milk would be condemned. There is without doubt room for a difference of opinion on the justice of this standard as applied to milks of a relatively high total bacterial content. On the whole, there can be little question that a larger percentage of the higher count milk is produced under unsatisfactory sanitary conditions than in the case of the milks of lower bacterial content. On the other hand, experimental work and practical experience have shown that a vast majority of the high counts in market milk are due to growth of the bacteria in the milk or on the utensils, rather than initial contamination with foreign material. Is the same true of the colon count? Such a conclusion seems logical on the basis of the known rapid growth of these organisms in milk which is not well cooled.

It has been suggested that since colon organisms are known to grow in milk, a standard should be adopted which would limit the number of these organisms in proportion to the total bacterial count, rather than applying one numerical standard for all milks. Since it was seen that a standard of less than 1,000 colon organisms per cc. does not appear unduly harsh upon the producers of milk containing less than 100,000 bacteria per cc., a logical suggestion would be to limit the colon count to one per cent of the total count. This was tried, with the results which are included in table 1. This standard, which condemned about the same proportion of the milks which contained less than 10,000 bacteria per cc. as of those

which bacterial counts above 300,000 per cc., would hardly appear reasonable.

TABLE 1
Application of various colon standards to 450 raw milks

TOTAL BACTERIAL COUNT	NUMBER OF SAMPLES	PERCENTAGE THAT WOULD BE CONDEMNED		
		100 per cc.	1,000 per cc.	1% of total
Less than 10,000 per cc.	102	11	0	16
10,000 to 100,000 per cc.	207	50	10	15
100,000 to 300,000 per cc.	63	73	33	25
300,000 or more per cc.	78	63	45	18

GROWTH OF COLON ORGANISMS IN MILK

It is well known that the colon bacteria, in common with microorganisms generally, grow much faster if the milk is not well cooled. There appears, however, to be a mistaken idea, fairly generally held, that organisms of this type will not grow in "properly cooled" milk. The statement has been repeatedly made that they wil not increase in milk held at 50° F. There is, however, no excuse for this mistaken impression, as the question was thoroughly answered by the excellent work of Ayers and Clemmer.[2] We have found that all of the well-known species of the colon group grow at 50° F., and, further, that the colon count of samples of milk held at this temperature increases. The practical nature of this fact is forcibly brought out when we consider the relative rates of growth of the colon organisms and the total bacterial population of milk.

Our studies at various temperatures have shown that the colon count in milk generally increases more rapidly than the total count during the presouring stage, which includes the whole period of interest in the fluid milk industry. This was true in a vast majority of the experiments conducted at 50° F. The same results were obtained, quite naturally, at 59° F., as well as at higher temperatures. The fact that the colon count usually increases in milk at 50° F. at a faster rate than the total count may well give pause to suggestions that a numerical colon standard be applied to milks of relatively high bacterial content. The results of these experiments are summarized in table 2.

THE COLON STANDARD UNDER KNOWN CONDITIONS

It should be of interest now to consider how well the colon test performs when studied under controlled conditions. The data obtained from 220 tests are summarized in table 3. In each test the total bacteria and the colon organisms were determined on the fresh milk (usually about six hours old) and again after the experimental holding period.

[2] Ayers, S. H., and Clemmer, P. W. U. S. Dept. Agriculture, Bul. 739. 1918.

TABLE 2

Increase in colon count as compared with total bacterial count in milks held at various temperatures

TEMPERATURE HELD	NUMBER OF EXPERIMENTS	PERCENTAGE OF SAMPLES IN WHICH COLON COUNT INCREASED MORE RAPIDLY THAN TOTAL COUNT		
		24 hours	*48 hours*	*72 hours*
50° F.	29	62	79	83
59° F.	42	76	86	86
		3 hours	*6 hours*	*15 hours*
70° F.	20	65	80	100
86° F.	20	55	90	

All of these 220 milks were of good quality with reasonably low bacterial counts, and all had colon counts below 1,000 per cc.; 173 of them had less than 100 colon bacteria per cc. Of the 220 samples, 87 contained less than 10,000 total bacteria per cc.; 119 contained from 10,000 to 100,000 per cc.; while only 14 gave plate counts of 100,000 or more bacteria per cc.

TABLE 3

Application of a colon standard of less than 1,000 per c.c. to "clean milks" before and after holding

TREATMENT	NUMBER OF EXPERIMENTS	PERCENTAGE THAT WOULD BE CONDEMNED	
		Before holding	After holding
50° F.—24 hours	23	0	13
50° F.—48 hours	23	0	61
*50° F.—72 hours	23	0	83
59° F.—24 hours	41	0	66
*59° F.—48 hours	41	0	100
70° F.— 3 hours	18	0	28
70° F.— 6 hours	18	0	67
*70° F.—15 hours	17	0	100
86° F.— 3 hours	16	0	88

* These treatments probably do not come within the realm of common practice but are included as a matter of interest.

A glance at table 3 will be sufficient to show the inadequacy of the colon test as an indicator of the initial sanitary quality of the milk. These experiments show that about two-thirds of these initially satisfactory milks which were held either at 50° F. for 48 hours or at 59° F. for 24 hours would have been condemned on the basis of containing more than 1,000 colon bacteria per cc. Of those held at 50° F. for only 24 hours, 13 per cent would have been judged below standard.

One argument remains to be answered. When the colon test is used, one not infrequently encounters two milks of about the same total bacterial

content, but one low and the other high in organisms of the colon group. The contention of those who advocate the test is that such a disparity signifies a true difference in sanitary quality; that the conditions surrounding the production of the milk with the high colon count must have been unsatisfactory. This point of view is well expressed in the following quotation from a letter written by a state health official:

> "I have already referred to the recent sample of ice cream which had a standard count per cc. of 2,000,000 and a colon count of 1,600,000. With this may be compared results upon two other samples, encountered a few days later, which had, per cc., standard counts, respectively, of 4,500,000 and 5,500,000 and colon counts of 15,000 and 5,000. Despite the high standard counts for the latter, if obliged to eat any of the three, I would select the two last referred to every time. I am yet to be convinced that the exceedingly high colon count in the first does not serve as evidence of an objectionable and possibly deleterious form of contamination."

Such an opinion seems reasonable, and one might well feel inclined to agree with it. On the other hand, our opinions, however logical, prove exactly nothing unless founded upon facts obtained under known conditions.

In table 4 are presented some data which illustrate forcibly the fallacy underlying the above argument. The results obtained with fourteen milks were selected and grouped in pairs. In each pair, the two milks when fresh gave practically the same counts for total bacteria and colon organ-

TABLE 4

The failure of the colon test as an indicator of the initial sanitary quality of milk

TREATMENT	SAMPLE NUMBER	BEFORE HOLDING		AFTER HOLDING	
		Total	Colon	Total	Colon
59° F.—24 hours	133	3,900	< 10	217,000	130
	149	3,500	< 10	66,000	4,200
59° F.—24 hours	137	19,500	< 10	680,000	50
	116	17,700	< 10	2,030,000	18,000
59° F.—24 hours	118	4,300	40	400,000	32,000
	135	4,200	50	80,000	790
50° F.—72 hours	78	3,200	< 10	1,120,000	2,480
	71	2,700	< 10	840,000	50
50° F.—24 hours	92	80,000	240	130,000	390
	83	63,000	200	540,000	4,500
70° F.— 6 hours	65	6,000	< 10	370,000	460
	63	6,000	< 10	630,000	3,500
59° F.—48 hours	129	18,700	20	106,000,000	26,000,000
	132	19,400	30	196,000,000	9,500

isms; after being held under identical conditions the results were in striking contrast.

These fourteen examples were selected from our data on 362 milks which were tested under known conditions. While such divergent results with two milks of apparently similar initial quality are not the rule, they are by no means exceptional. Our records contain many more equally striking examples. Such results certainly prove beyond any question that the colon test is not a reliable index to the initial bacteriological condition of milks in which bacterial growth has taken place.

THE COLON TEST FOR HIGH GRADE MILK

The fact that the colon test is not applicable to raw market milks in which bacterial growth is not excluded should not blind us to its possible value for high grade milk of low bacterial content. Again it should be repeated that organisms of the colon group are not only undesirable in milk, but they gain access to it from sources which are objectionable.

Most of the 102 milks examined which contained less than 10,000 total bacteria per cc. were also very low in colon organisms. The distribution of these milks with respect to their colon counts is shown in table 5.

TABLE 5

The number of colon organisms in milks containing less than 10,000 bacteria per cc.

NUMBER OF SAMPLES	PERCENTAGE OF SAMPLES THAT CONTAINED LESS THAN			
	10 per cc.	25 per cc.	100 per cc.	1,000 per cc.
102	48	73	89	100

From these results it would appear that if the colon count is used as a supplementary test on milks containing less than 10,000 total bacteria per cc., a limit of 100 colon organisms per cc. probably would not prove unreasonable, while a limit of 1,000 per cc. appears to be too lenient.

The American Association of Medical Milk Commissions recommends a colon test for certified milk "at frequent intervals." It is stated that certified milk should contain less than 10 colon organisms per cc. In view of the fact that 48 per cent of the milks containing less than 10,000 bacteria per cc., produced by merely "good" farmers and sold at ordinary prices, had colon counts of less than 10 per cc., it would seem that certified milk should have little difficulty in meeting this standard. Ayers and Clemmer, in the examination of fourteen samples of certified milk, as delivered, found three which contained 10 or more colon organisms per cc., but the highest colon count was only 20.

In this discussion of the application of the colon test to high grade milks, we have resorted more to practical experience with the test than to rigid

scientific studies under controlled conditions. Definite conclusions should not be drawn without more data on high grade milks produced under known circumstances with respect to sanitation.

With respect to the application of the colon count to milks of low bacterial content, a word of warning is not out of place. It has been clearly shown that this test is misleading when used for milks in which bacterial growth has taken place. There have been regulations which prescribed, for special raw milks, a total bacterial content of less than 25,000 per cc. and a colon count of less than 100. Among milks which range in bacterial count between 10,000 and 25,000 per cc., there are apt to be many in which some growth has taken place, if not in the milk itself, at least on the utensils. It might be contended that such a small amount of growth would not invalidate the results obtained with the colon test. Our results on this point are of some importance. Of 88 milks which contained between 10,000 and 25,000 bacteria per cc., 29 per cent had colon counts of more than 100 per cc. It is not likely that such a large proportion of these relatively high grade milks were produced under conditions which would be considered insanitary according to present standards. The important point to remember is that the colon count usually increases at a more rapid rate than the total count. The practical significance of this fact can not be emphasized too strongly.

CONCLUSION

In view of the facts, the conclusion seems inescapable that the colon test, as now used and advocated in some localities, is of no especial value as an index to the sanitary conditions surrounding the production of raw milks of usual market grade. This conclusion applies to all raw milks except those which are so handled that bacterial growth is prevented.

In the case of high grade raw milks containing less than 10,000 total bacteria per cc., the colon test may have a place as a supplementary index of quality. If it is used for such milks, a standard of less than 100 per cc. would not seem to be unreasonable.

For certified milk, the standard of less than 10 colon organisms per cc. recommended by The American Association of Medical Milk Commissions does not appear unduly stringent.

If the time should arrive when all milk is required to be promptly cooled to 45° F., and maintained at this temperature, the colon test might well prove to be of more general value.

American Dairy Science Association Announcements

ANNUAL MEETING

JUNE 26–29, 1933, AT THE UNIVERSITY OF ILLINOIS, URBANA, ILLINOIS

The Executive Committee has chosen the University of Illinois, Urbana, Illinois, as the place to hold the 1933 annual meeting of the American Dairy Science Association. The dates for the meeting are June 26–29, inclusive.

June 26 will be devoted to conferences and demonstrations in the teaching of the judging of dairy cattle and dairy products. Although this day will be of special interest to those engaged in the coaching of teams, the sessions will be open to all members. The regular program will start on the 27th and continue for three days. This program will consist of short papers. Each speaker will be limited to fifteen minutes for the presentation and discussion of his paper.

Members desiring to present papers should submit subjects to the Chairman of the Program Committee before April 15. Papers may cover new unpublished results of research or discussions of teaching and extension methods.

Urbana may be reached by automobile or by the Illinois Central, Big Four, and Wabash railroads. Arrangements are being made to house visitors in dormitories at a minimum expense, although excellent hotel facilities are available for those preferring them.

The Century of Progress Exposition in Chicago is about a three hours' drive from Urbana on good concrete roads. It is hoped that many will plan on bringing their families and attend both the meeting in Urbana and the Exposition.

The Program Committee consists of H. A. Ruehe, of Urbana, Illinois, Chairman; C. L. Roadhouse, of Davis, California, and Fordyce Ely, of Lexington, Kentucky.

ASSOCIATION NEWS

DR. PHILIP RUPP retired from active service as dairy chemist in the Bureau of Dairy Industry on December 31, 1932. Dr. Rupp is author or coauthor of numerous publications on the chemistry and bacteriology of milk. Physician, Chemist; born New York City, N. Y., Dec. 22, 1862; Ph.B., School of Mines, Columbia University, N. Y., 1884; M.D., College of Physicians and Surgeons, Columbia University, 1887; Ph.D., University of Freiburg, Baden, Germany, 1901; practicing physician, New York City, 1887–

97; chemist, Farbenfabriken, v. F. Bayer, Elberfeld, Germany, 1901–02; chemist, C. F. Boehringer & Sohne, Waldhof-Mannheim, Germany, 1902–04; chemist, Bureau of Chemistry, U. S. Dept. of Agriculture, 1908–09; Dairy Chemist, Dairy Division, B. A. I., U. S. Dept. of Agriculture, and Bureau of Dairy Industry, U. S. D. A., since 1909.

Richard C. Munkwitz

Mr. Richard C. Munkwitz, Associate Professor of Dairy Manufacturing, University of Maryland, College Park, Maryland, died Friday, January 6, 1933, in the Naval Hospital, Washington, D. C., of a complication of ailments. Professor Munkwitz, who was 38 years old, was born in Edgar, Wisconsin.

During the World War he served as a non-commissioned officer with the 339th Infantry in the Archangel Expedition in Russia. He was a member of the National Farm House Fraternity and the Masons.

Professor Munkwitz received his college training at the Universities of Wisconsin and Illinois. He graduated from the University of Wisconsin with his Bachelor's Degree in 1922, and the following year received his Master's Degree from the University of Illinois. He came to the University of Maryland in 1925 as Assistant Professor of Market Milk.

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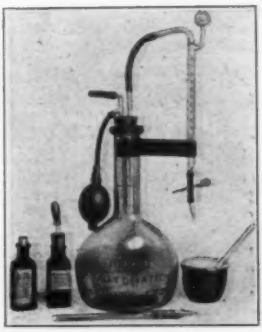

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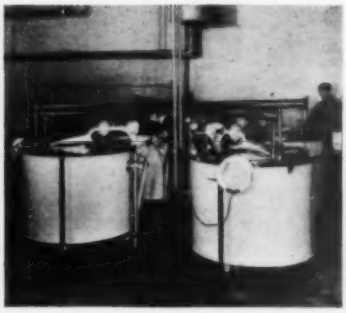

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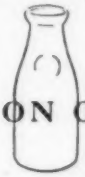


WHY TAKE CHANCES ON OFF-FLAVORED MILK?

Play Safe with the only pasteurization temperature control that's variation proof

If pasteurization conditions never varied, any temperature controller would do.

But conditions *do* vary, *plenty* and *suddenly* — hence the CP Dual Controller System, standard equipment on the Precision Heater.

Happen what may out in your plant, this sensitive electric "brain" sees that your final milk temperature keeps well within those narrow limits between the legal minimum and the flavor danger-line.

Your operator can change suddenly from quarts to pints or back again, shut down the heater completely, switch from milk to heavy cream, receive milk at *any* initial temperature—and still assure your critical trade perfect natural flavor and creaming quality in every bottle.

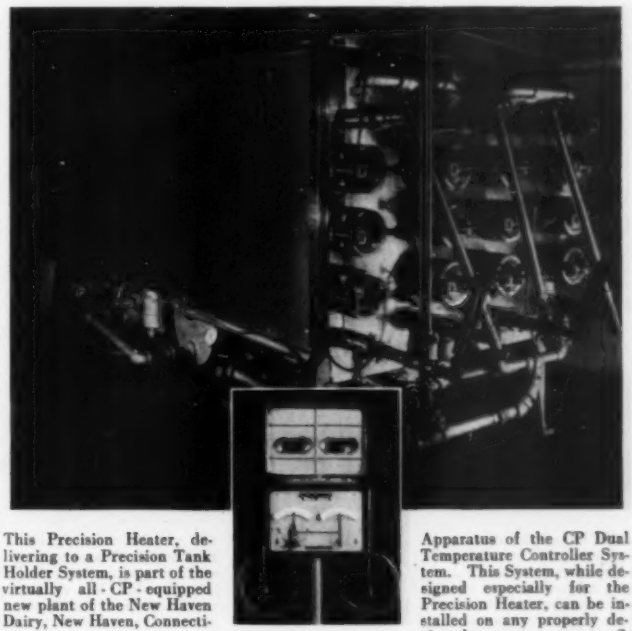
Variations in pasteurizing temperature under *all* operating conditions may be held to within three tenths of one degree.

Here's how and why, in simple language: The CP Dual *anticipates* temperature change. A bulb one-third the way back through the milk line of the heater offsets any disturbance before it can play hob with the final heating temperature. That's why the Dual remains absolutely unique — with no counterpart of any make on the market.

This system is only one of many features of the Precision Heater. In its five years of wide use, many refinements naturally have evolved. Now more compact, more accessible, better proportioned than ever. Choice of materials and finish. Sized for the moderate plant, too — down to 7,000 pounds per hour.

Ready to attach to your water, steam and power lines and go to work next week. So easy to play safe! May we tell you more?

THE CREAMERY PACKAGE MFG. COMPANY, 1243 W. Washington Blvd., Chicago, Ill. Branch offices in principal cities.



This Precision Heater, delivering to a Precision Tank Holder System, is part of the virtually all-CP-equipped new plant of the New Haven Dairy, New Haven, Connecticut.

Apparatus of the CP Dual Temperature Controller System. This System, while designed especially for the Precision Heater, can be installed on any properly designed counter-current, 2-stage heater.

CP *Precision* Heating

With CP **Automatic** *Dual Controller System*

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Electric Sterilization

Maintains Laboratory Standards

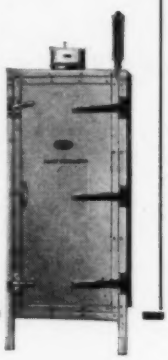
THE ESCO Electric Dairy Utensil Sterilizer brings to the laboratory a more simple method, better in every way than those in ordinary use . . . dependable, under perfect control and adequate for almost every need. Sterilization without the bother of chemicals or steam, and with seams, dents or scratches barren to new cultures.

Proved by test, the ESCO lends itself particularly well to laboratory use in the sterilization of vessels used in culture work.

Bulletin No. 612, N. Y. Experiment Station (Geneva) says that 200 degrees F. in an ESCO Sterilizer for 10 minutes "was sufficient to kill all bacteria of the colon-aerogenes group, dry the utensils and to reduce bacterial contamination to insignificant numbers." Technical Paper No. 553, Pa. State College says, of the ESCO Electric Sterilizer, "It is one of the most convenient methods of sterilizing insofar as the farmer is concerned."

Descriptive literature sent gladly upon request.

ESCO CABINET COMPANY, Manufacturers, West Chester, Pa.



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1. Cleaner, more sanitary conditions.
2. Longer life for equipment.
3. Important savings in time and money.
4. Better dairy products.

The use of Wyandotte in dairy cleaning insures sanitary cleanliness with minimum effort and expense.

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THE J. B. FORD COMPANY
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Bacto-Trypsin Digest Agar is a new medium for the isolation and propagation of Lact. acidophilus. It may be used with utmost satisfaction in determining the number of viable organisms in acidophilus products. The medium is readily prepared for use in the laboratory and assures comparable results.

The formula was suggested by Dr. H. A. Chaplin.

BACTO-YEAST DEXTROSE AGAR

This is an excellent medium for the enumeration of pin-point colonies in milk and other dairy products. It is of invaluable assistance in controlling the sanitary conditions of dairy plants. Neither filtration of the solution nor adjustment of its reaction are required.

The formula used is identical with that recommended by the Committee on Bacteriological Methods of Analyzing Dairy Products of the American Dairy Science Association. The medium is recommended by the Committee for investigations and research in the determination of thermophilic organisms. (*Journal Dairy Science*, 15, 383, 1932.)

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